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GROWTH HORMONE RECEPTORS AND BINDING PROTEINS IN TISSUE GROWTH HORMONE RESISTANCE

BY

KERRY LYNN HULL

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A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY
EDMONTON, ALBERTA
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To Mom, Dad, Carmen, and John with love

ABSTRACT

Growth hormone (GH) is critically involved in growth and metabolism. However, some tissues are unresponsive to GH and certain inherited growth disorders are characterised by a resistance to GH action. As GH requires target tissue receptors to mediate its effects, a GH receptor (GHR) defect could account for this GH unresponsiveness. This hypothesis was therefore investigated.

Tissue GH-resistance was assessed in avian thyroid and pituitary glands, which have traditionally been considered to be GH-unresponsive. However, GHR gene expression was demonstrated in both tissues and in a tumorous pituitary (GH_3) cell line by Northern blotting and by the polymerase chain reaction. Moreover, GHRs and/or GH-binding proteins (GHBPs; derived from the extracellular domain of the GHR) were also detected by radioligand binding, Western blotting, immunocytochemistry and/or radioimmunoassay. GH resistance in these tissues does not therefore result from a GHR deficiency, although the actions of GH in these sites may be antagonized by competitive ligand binding to GHBPs, that lack signal transduction systems.

Genetic GH-resistance was investigated in sex-linked dwarf (SLD) chickens and guinea pigs. GHR mRNA of normal size and composition was found to be abundantly present in hepatic and non-hepatic tissues. Moreover, despite an absence of hepatic GH-binding activity, hepatic GHRs of normal molecular size were demonstrated in SLD chickens. Proteins capable of binding I^{125}-oGH were also detected in SLD serum, thus hepatic and serum GH-binding moieties may be independently derived in chickens. Conversely, abundant GH-binding activity is present in guinea pig liver but not serum, although serum GHBP-immunoreactivity was abundantly present. Guinea pig GHBPs may also be derived independently of hepatic GHRs, since a truncated (1.9kb) transcript was detected that may encode a GHBP structurally distinct from the GHR. This serum GHBP was immunoreactive with MAb 4.3, raised against the unique hydrophilic sequence of rat GHBP, and thus closely resembles soluble GHBP-like proteins rather than membrane receptors. This GHBP may, however, be unable to bind GH. GHR defects rather than a GHR deficiency are thus likely to account for the GH-resistance in these hereditary disorders.

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LIST OF ABBREVIATIONS

Analysis of Overall Variance (ANOVA)

Association Constant (Ka)

Base Pairs (bp)

Binding Capacity (Bmax)

Bladder (blad.)

Complementary Deoxyribonucleic Acid (cDNA)

Complementary Ribonucleic Acid (cRNA)

Correlation Coefficient (r)

Day (d)

Deoxyribonucleic Acid (DNA)

Diacylglycerol (DAG)

Diiodothyronine (T_2)

Growth Hormone (GH)

Growth Hormone-Placental Variant (GH-V)

Growth Hormone Binding Protein (GHBP)

Growth Hormone Receptor (GHR)

Growth Hormone Receptor - Third Exon Deleted (GHR3d)

Growth Hormone Receptor - Short Form (GHR_{sh})

Growth Hormone Releasing Factor (GRF)

Hour (h)

Inositol Triphosphate (IP_3)

Insulin-like Growth Factor (IGF)

Kilodalton (kDa)

Laron Syndrome (LS)

Laron-type Dwarf (LTD)

Messenger Ribonucleic Acid (mRNA)

Minute (min)

Molecular Weight (MW)

Monoclonal Antibody (MAb)

Muscle (musc.)

Normal Cornell Strain (K strain)

Non-specific Binding (NSB)

Pituitary Gland (pit.)

Polyclonal Antibody (p(Ab))

Polymerase Chain Reaction (PCR)

Radioimmunoassay (RIA)

Recombinant Chicken Growth Hormone (rcGH)

Recombinant Rat Growth Hormone Binding Protein (rGHBP)

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Ribonucleic Acid (RNA)

Sex-linked Dwarf Strain (SLD)

Somatostatin (SRIF)

Spearman Rank Correlation (r_s)

Standard Error of the Mean (SEM)

Student's T-Test (t)

Thyroid Hormone Releasing Hormone (TRH)

Thyroid Stimulating Hormone (TSH)

Thyroxine (T_4)

Total Binding (B_o)

Transfer Ribonucleic Acid (tRNA)

Transmembrane Domain (TM)

Triiodothyronine (T_3)

Weight/Volume (w/v)

CHAPTER 1: LITERATURE REVIEW

1.1. INTRODUCTION

A. Hormones and Communication

Multicellular organisms require intercellular communication systems for integrative homeostatic control. Communication between cells is accomplished by a number of informational molecules that are released into extracellular space from one cell and influence the activity of other cells. "Hormones" are chemical messages derived from protein or steroidal precursors that provide regulatory information as a result of their interaction with receptor molecules in target cells. This information is then relayed into a physiological response through the intervention of intracellular signal transduction systems. The role of receptors, therefore, is two-fold; to recognize a specific signal, and to induce an appropriate action in response to that signal.

Hormonal receptors may be ubiquitously distributed within the intracellular compartments of target sites or located within the plasma membrane and exposed to the extracellular milieu. Generally, hydrophilic hormones (eg. steroids, iodothyronines), which are capable of passing through the cell membrane, interact with cytosolic and nuclear receptors, whereas hydrophobic hormones (peptides) remain in the extracellular space and interact with membrane receptors. This is, nevertheless, an oversimplification, since it is now known that peptide hormones can interact with intracellular and nuclear receptors and steroid hormones possess receptors in the plasma membrane and may also act by changing membrane fluidity. The receptors for protein hormone receptors are the focus of this review.

B. Historical Background - The Receptor Concept

The importance of receptors in hormonal action was recognized early in the 20th century by the pharmacologists Langley (1906) and Ehrlich (reviewed in Ehrlich, 1956). These scientists proposed that biologically active molecules (such as nicotine and tetanus toxin) interact with specific regions of the cell surface ("receptive substances") that are

important in signal mediation. The importance of membrane receptors was subsequently demonstrated by the ability of proteases to reduce cellular responsiveness to peptide hormones, as these enzymes can degrade membrane-bound receptors projecting into the extracellular milieu but cannot enter viable cells (Kono, 1969). Moreover, since antibodies directed against specific hormones interfere with hormone actions that have already been initiated (Pastan *et al.*1966), cellular entry is thus not a requirement for hormonal action. The spatial separation between the hormone binding-sites in the plasma membrane and the intracellular sites of hormonal action therefore necessitates the intervention of intracellular messengers.

The first intracellular (so-called "second messenger") system was characterised by Sutherland and colleagues (Rall *et al.* 1957; Robinson *et al.* 1971), who demonstrated roles for cyclic adenosine monophosphate (cAMP) in mediating hormonal action. Cyclic guanidine monophosphate (cGMP), calcium, diacylglycerol (DAG), inositol phosphates, prostaglandins and nitric oxide have since been found to have similar roles as second messengers. Receptor-mediated signal transduction was, therefore, established as a concept for understanding the mechanism of protein hormone action by 1971. At that time, the existence of receptors was directly demonstrated by Lefkowitz (1970), who detected binding sites for I^{131}-labelled hormones (such as ACTH) on plasma membranes.

C. Hormone Receptors: Definitions

Traditionally, hormone receptors have been differentiated from other proteins by a number of criteria (Beaulieu, 1990). Namely, the ligand-receptor interaction must induce a biological response, and this interaction must be specific, reversible and saturable. Proteins that bind hormones but do not induce biological responses are considered to be binding proteins rather than receptors (Kahn, 1989). Alternatively, in contrast with signal-mediating "type I" receptors, a subclass of receptors ("type II" receptors) are not involved in signal transduction but mediate ligand internalization (Kaplan, 1981). Classical hormone receptors have also been defined as having a limited regional distribution. However, the techniques of molecular biology have recently demonstrated that many receptors are found ubiquitously throughout the body, albeit in

differing amounts. It is also now known that cellular receptors are not obligatory for receptor-mediated action, since biological responses can be induced in cells deficient in specific receptors. This phenomenon, described as "receptor mediated action without receptor occupancy"(Fletcher & Greenan, 1985), is thought to result from the passage of second messengers through gap junctions from adjacent, receptor-containing cells.

D. Hormone Receptors: Mechanisms of Action

Receptor-mediated action requires hormone recognition and the induction of a cellular response. The latter "effector" function involves activation of a cascade of events (often phosphorylation and activation of cellular kinases) that results in altered enzyme activity, protein synthesis and/or other physiochemical changes in cellular structure and function (Figure 1.1)(Kahn, 1989). Initiation of this cascade can be accomplished by the receptor itself or by associated membrane proteins. For instance, insulin binding results in autophosphorylation and activation of a tyrosine kinase located within the insulin receptor. This activated kinase is capable of phosphorylating and activating other cellular kinases (the second messengers), and the resulting "kinase cascade" culminates in a cellular response (Kahn, 1989). Other receptors contain or are linked to ion channels, and hormone binding increases or decreases channel permeability (Kahn, 1989). However, most hormones act via separate effector protein(s) that are usually linked to membrane enzymes, such as adenylate cyclase and phospholipases. These linking proteins (or G proteins) induce (G_s) or inhibit (G_i) the activity of membrane enzymes that are responsible for the generation of second messengers such as cAMP, DAG and IP3. These messengers, in turn, activate protein kinases: protein kinase A by cAMP, and protein kinase C by DAG (Kahn, 1989).

The cascading nature of these intracellular signalling pathways permits rapid signal amplification, as a single hormone-receptor complex can activate numerous second messengers. Moreover, each second messenger molecule can activate a protein kinase that in turn activates or induces the synthesis of many intracellular proteins. These proteins may even reduce or inhibit the activity of other second messenger systems within individual cells and regulate the receptor-effector-signal transduction systems of

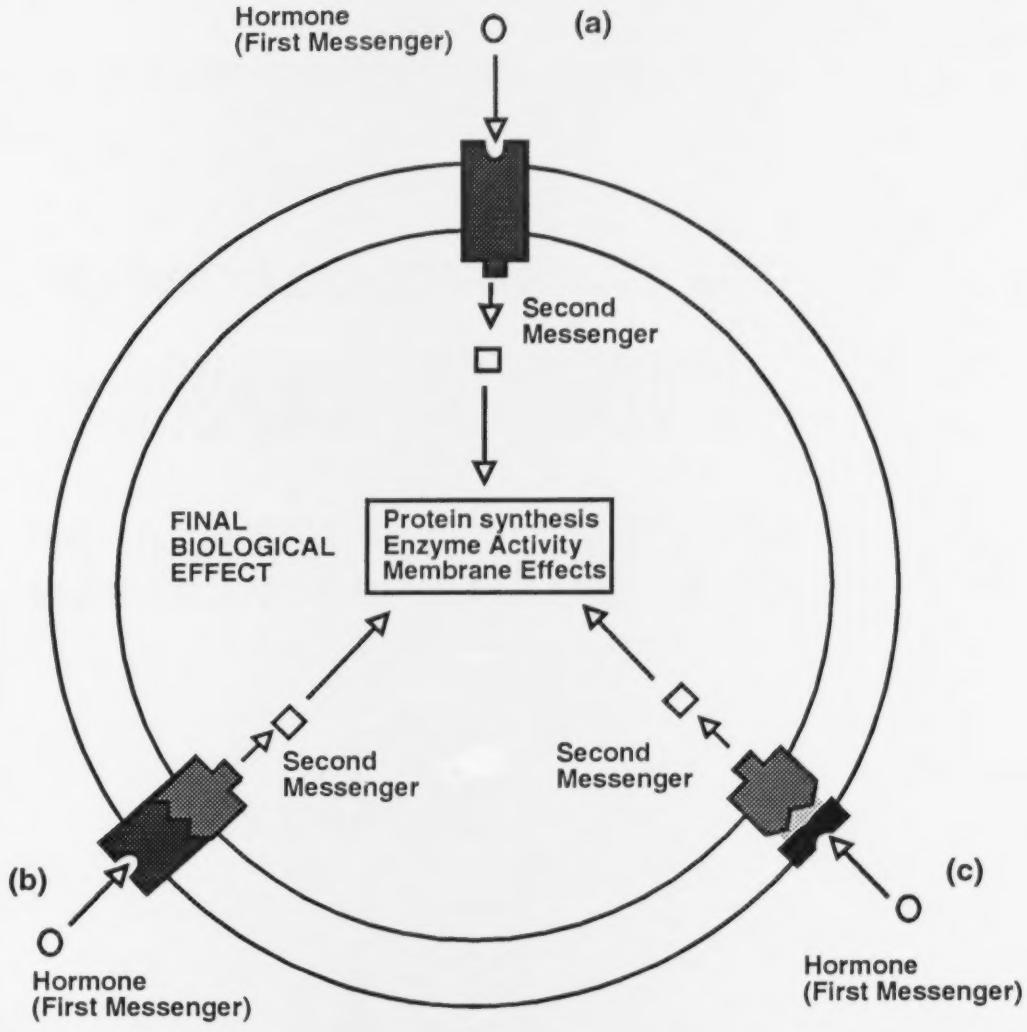

Figure 1.1. Models of peptide hormone action. The hormone-binding site (receptor) and signal-transmission site (effector) may be contained in a single molecule (a), in two interacting molecules (b) or in two molecules coupled by a third molecule (c). Tyrosine kinase receptors are examples of the first type. The IL-6 receptor coupled to the signal-tranducing gp130 protein may be a representative of the second type (Stahl and Yancopoulos, 1993). Hormone receptors linked to adenylate cyclase (via G-proteins) are examples of the third type.

heterologous ligands. "Cross-talk" between signal transduction systems therefore provides subtle control over the cellular response to individual and multiple hormonal signals.

The cellular response to multiple hormones may be mediated in cells by a single signal transduction system, acting as a final-common-pathway for the induction of identical actions by unrelated hormones. A single second messenger system may also mediate divergent actions of a single hormone in different cells, depending upon the genetic complement of individual cells.

E. Hormone Receptors: Properties

The physiochemical properties of receptors and the kinetics of ligand dissociation are well-established and exhaustively reviewed (Baxter & Funder, 1979; Kahn, 1989; Kaplan, 1981; Beaulieu, 1990; Viru, 1991). A brief summary of these concepts is present below.

i. Binding Kinetics: The reversibility and non-covalent nature of the hormone-receptor interaction permit analysis of binding kinetics. In a system at equilibrium, binding can be conceptualized by the simple enzymatic equation

$$[H] + [R] \underset{k_d}{\overset{k_a}{\rightleftharpoons}} [HR]$$

(H; free hormone, R; free receptor, HR; hormone-receptor complexes, k_a; association constant, k_d; dissociation constant). In general terms, this equation mathematically states that the amount of bound hormone depends on the concentrations of unbound receptor and free hormone. The amount of bound hormone (B) at various concentrations of free hormone (F) can be graphed directly, but these data are usually transformed into Scatchard or Lineweaver-Burk plots. In Scatchard analysis, which is the most frequently used, B/F is plotted on the y-axis and B is plotted on the x axis. If the hormone-receptor interaction involves only a single receptor type, the slope of this graph will define receptor affinity (Ka) and the x-intercept will define receptor capacity (Bmax).

This figure has been removed for copyright purposes. The original figure can be found in: Kahn, C.R. (1989) Membrane receptors for peptide hormones. In: *Endocrinology* (Edited by L.J. DeGroot) W.B. Saunders, Toronto, p. 6-16.

Figure 1.2. A. Coupling between receptor occupancy and biological effects. Curve "a" illustrates strict linear coupling, curve "b" represents non-linear coupling and curve "c" illustrates nonlinear coupling plus spare receptors. B. The relationship between sensitivity and responsiveness. Hormone resistant states can be associated with a rightward shift of the dose-response curve (decreased sensitivity), a decrease in maximal response (decreased responsiveness) or a combination of the two. Decreased sensitivity indicates a defect at a non rate-limiting step (often the receptor) whereas decreased responsiveness indicates a defect at a rate-limiting step (usually post-receptor). Independent and dependent increases in sensitivity and/or responsiveness may also occur. Based on Kahn, 1989.

ii. Receptor Occupancy and Cellular Responses: The close relationship between changes in receptor occupancy and cellular responses is an important characteristic of the hormone-receptor interaction (Figure 1.2.A). Theoretically, a linear relationship should exist between receptor occupancy and biological effect. Thus, a half-maximal response should occur when 50% of receptors are occupied. However, this relationship is rarely linear in physiological hormone-receptor systems, as half-maximal responses are often observed at occupancy levels below 50%. Moreover, maximal responses to a hormone can be obtained when a large number of receptors (spare receptors) remain unoccupied. This overabundance of receptors has important implications for the kinetics of hormonal action, as it amplifies the biological response to a given hormone concentration. As shown in Figure 1.2.B, a reduction in the number of spare receptors (a decrease in sensitivity) shifts the dose-response curve to the right, thus a larger hormone concentration is required to initiate an identical cellular response. However, the maximal biological response (responsiveness) is unaltered, as enough receptors remain to saturate signal transduction systems. Conversely, a defect in post-receptor signalling reduces responsiveness regardless of receptor number, as intracellular signalling is often the rate-limiting step (Kahn 1989). Sensitivity and responsiveness can also be increased (respectively) by receptor upregulation and by a sensitization of the signal transduction pathway.

iii. Receptor Mobility: Receptor-mediated action is also dependent upon adequate receptor mobility, as receptors move within the fluid membrane bilayer as well as from the membrane to intracellular compartments. Lateral receptor movement within the membrane permits interactions with other receptors and effector molecules. Lateral mobility also enables hormone-receptor translocation into intracellular compartments by receptor-mediated endocytosis. As shown in Figure 1.3, hormone-receptor complexes aggregate in uncoated or coated pits and are internalized. Internalization can result in degradation of the hormone and/or receptor, recycling of the receptor to the membrane, re-release of the hormone into the extracellular milieu or hormone delivery to intracellular sites of action (Figure 1.3).

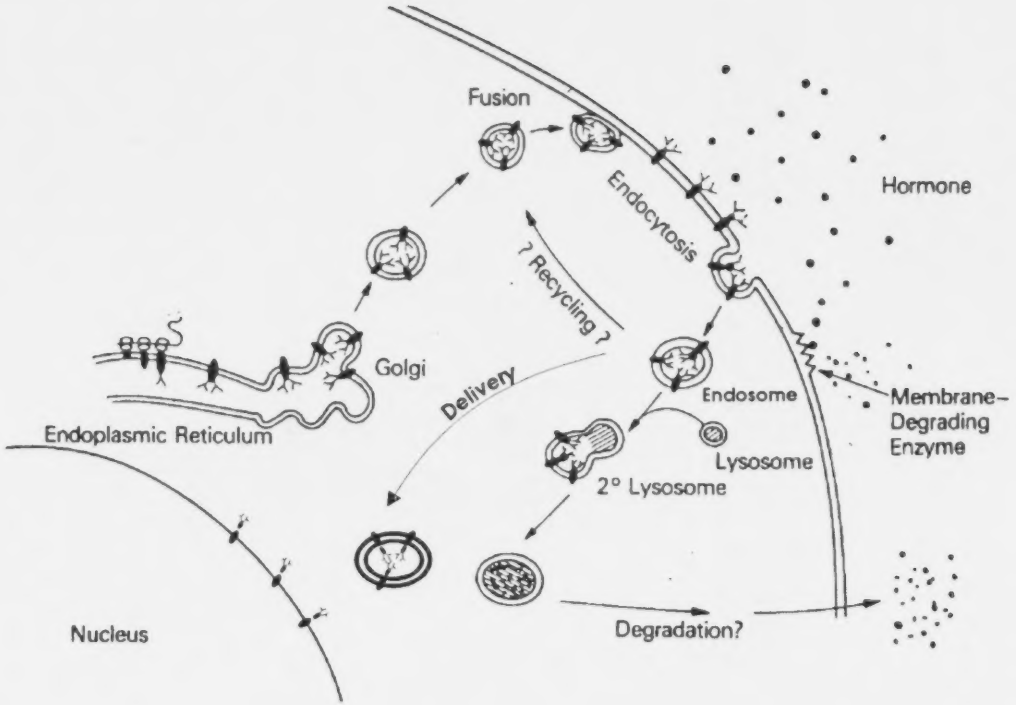

Figure 1.3. A general model for the life cycle of the peptide hormone receptor. Membrane receptors are synthesized in the rough endoplasmic reticulum, processed in the Golgi apparatus and inserted into the membrane. After specific binding to hormone molecules, the receptors aggregate and are internalized by either coated or noncoated pits. The endosome containing the hormone-receptor complex may fuse with lysosomes, resulting in degradation of both the hormone and receptor, the receptor may be recycled to the membrane and the hormone degraded, or the hormone may be rereleased into the extracellular space. Internalization may also deliver hormones to intracellular sites of action. Based on Kahn, 1989.

iv. Binding Specificity: In theory, receptors must be highly selective, as they must distinguish one ligand from thousands of others. However, in practice, receptors are often promiscuous and will bind to hormones that resemble their specific ligand. The affinity of this heterologous interaction depends on the structural similarities between hormones. For instance, human GH and prolactin are highly similar in structure, and both hormones bind a "lactogenic GH receptor" with high affinity.

v. Receptor Regulation: The amount and affinity of hormone receptors on the cell surface is not constant. Rather, receptor levels are increased (upregulated) or decreased (down-regulated) in response to homologous and heterologous stimuli. These changes in receptor binding characteristics allow the cell to respond to minute alterations in hormone concentrations. Moreover, the receptor down-regulation that occurs in the presence of high concentrations of hormone protects the cell from excess stimulation. Hormones can also regulate the abundance and affinity of the receptors of other ligands, thus permitting integration of hormonal responses.

Receptor levels can be regulated by alterations in protein synthesis or by modification of receptor trafficking. For instance, binding of some hormones (eg. LHRH, GH) results in internalization of the hormone:receptor complex and a reduction in membrane binding-sites. Thus, most hormones acutely down-regulate their own receptors. It is also possible that receptor number may be acutely down- or up-regulated by modulating receptor mobilization from intracellular pools. However, chronic alterations in receptor number are generally mediated by alterations in protein synthesis.

F. Receptor Dysfunctions

Alterations in receptor function can result in increased or decreased tissue responsiveness and are associated with numerous disease states (Table 1.1)(reviewed by (Laycock, 1978; Mendelson, 1988; Smith *et al.*1988; Johnson & Dhanasekaran, 1989; Spiegel, 1989; Carson-Jurica *et al.*1990; Taylor *et al.*1990; Lee *et al.*, 1991; Spiegel *et al.*1992; Wong *et al.*1992; Cone & Mountjoy, 1993; Culig *et al.*1993; ; Refetoff *et al.*1993). Excessive receptor activity is often phenotypically indistinguishable from

Table 1.1. Disease states associated with receptor dysfunctions.

HYPOACTIVITY SYNDROMES		
Steroid Receptors		
Vitamin D-resistant (Type II) Rickets	Vitamin D_3 receptor	ligand-binding domain DNA-binding domain stop mutation
Testicular Feminization	androgen receptor androgen receptor androgen receptor	receptor number receptor stability DNA-binding domain
Generalized thyroid hormone resistance	T_3 β receptor	ligand-binding domain receptor gene deletion stop mutation
Partial thyroid hormone resistance (PTHR)	sometimes T_3 β receptor	ligand-binding domain
Pseudo PTHR	T_3 receptor?	down-regulation?
Progesterone resistance	progesterone receptor	ligand binding domain
Other receptors		
Myasthenia gravis	nicotinic ACh receptor	receptor antibodies (inhibitory antibody) (increased receptor degradation)
G-Protein-linked Receptors		
Allgrove's Disease	ACTH receptor	
retinitis pigmentosa	rhodopsin	
nephrogenic diabetes insipidus	V_2 vasopressin receptor	
familial ACTH insensitivity pseudohypopara-thyroidism	ACTH receptor Gs-α	transmembrane domain deficiency
Albright hereditary dystrophy	Gs-α	deficiency

Tyrosine Kinase Receptors

Insulin-resistant diabetes mellitus	insulin receptor	stop mutation ligand-binding domain receptor processing receptor targetting accelerated degradation decreased signal transduction blocking antibodies (receptor downregulation)

HYPERACTIVITY SYNDROMES

Graves Disease	TSH receptor	receptor antibodies (potentiating antibody)
Somatotroph tumors	Gs-α	mutation, constitutive activation
Thyroid carcinoma	Gs-α	mutation, constitutive activation
	Gs-α	increased expression
McCune-Abright Syndrome	Gs-α	mutation, constitutive activation
Prostatic carcinoma	androgen receptor	point mutation-conveys adrenal androgen and progesterone responsiveness
Brucellosis	Go-α,Gi-α	ADP-ribosylation, uncoupling from receptor constitutive receptor activation
cholera	intestinal Gs-α	ADP-ribosylation, chronic activation

excessive hormone activity, and can arise from autoreceptor antibodies mimicking the ligand and constitutively activating the receptor (eg. Graves disease). An equivalent situation can arise from defects in signal transduction. A number of G-proteins serve as signal transducers for protein hormones, and genetic mutations or post-translational alterations in stimulatory G-proteins can also cause constitutive activation of the signal transduction pathway regardless of the presence or absence of ligand. Mutations that disable inhibitory G-proteins cause a similar hyperendocrine state. Finally, as shown in some prostatic cancers, point mutations in ligand-binding domains of receptors can convey responsiveness to heterologous ligands, thereby increasing receptor activation.

Receptor-mediated action can also be disrupted at a number of stages, resulting in phenotypic changes analogous to hormone deficiency. The majority of identified lesions in hormone resistant-states consist of point mutations or deletions in the ligand-binding domain. Curiously, the magnitude of ligand-binding impairment does not always correspond to the degree of phenotypic impairment. For instance, a reduction in the affinity of βreceptors for thyroid hormones is more detrimental than a complete lack of these binding sites (Refetoff *et al.*1993). The ability of a cell to respond to a particular hormone is also dependent upon a minimal level of receptors present in the appropriate intracellular compartment; thus, defects in receptor regulation, stability or targetting also result in varying degrees of hormone resistance. Defective signal transduction systems also block receptor-mediated actions. Identified lesions in this category include mutations in the DNA-binding domain for steroid hormones and G-protein dysfunctions for G-protein-linked receptors. Mutations in the tyrosine kinase portion of the insulin receptor have also been identified.

G. Hormone Receptors: Detection

The original method to detect membrane receptors, the "radioreceptor assay", remains a cornerstone of receptor research (Lefkowitz *et al.*, 1970). In this assay, a homogenate or membrane preparation from a tissue containing the receptor of interest is incubated with a labelled hormone or "ligand", which enables the formation of hormone-receptor complexes. Bound and free hormone are then separated by physicochemical

methods such as immunoprecipitation. The quantity, specificity and affinity of a receptor can be determined by this method. Antibodies can also be used to study receptors independently of ligand binding ability. Techniques such as immunoprecipitation, Western Blotting and radioimmunoassay are now extensively used to quantify, identify and characterize protein receptors, even if the receptor is defective and cannot bind its ligand. Immunological and functional assays have recently been incorporated into a ligand- immunofunctional assay (LIFA), which employs antibodies to both the ligand and the receptor to quantify functional receptors (Mercado *et al.*, 1993). Lastly, the techniques of molecular biology are now commonly used to study the genomic and transcriptional precursors of the receptors. Techniques such as Northern and Southern Blotting and the Polymerase Chain Reaction have been invaluable in identifying receptor mutations, sites of synthesis and regulation of receptor production.

1.2 GROWTH HORMONE RECEPTORS

Unlike most pituitary hormones, growth hormone (GH) lacks specific target cells and has widespread effects in all tissue and organ systems. These actions are mediated *a priori* by GH receptors (GHRs), which have a heterogeneity of structure and intracellular distribution and which may act by divergent signal transduction mechanisms (previously reviewed by Harvey & Hull, 1994b). The extracellular domain of the GHR also exists as a soluble GH binding protein (GHBP), which can enhance or inhibit GH action. In this section the synthesis, structure, and localization of GHRs and GHBPs are reviewed.

A. The Hematopoietin Receptor Superfamily

The growth hormone receptor (GHR) belongs to the hematopoietic receptor (HR) superfamily, which also includes the receptors for prolactin and numerous cytokines (eg. interleukins (IL)2-7, IL9, and IL11, erythropoietin, interferons, ciliary neutrophic factor (CNF) and others)(Bazan, 1989; Hochberg *et al.*, 1991a). Receptors of this family are type I glycoproteins with an extracellular, ligand-binding N-terminus, a single transmembrane domain, and an intracellular domain of variable length, and they are also present as truncated, soluble binding proteins (eg. GHR, Trivedi & Daughaday, 1988;

CNF, Davis *et al.*, 1991). Although the overall sequence homology between family members is suprisingly low, structural characteristics are conserved in a number of regions crucial for protein folding and/or formation of the binding pocket (Bazan, 1989) These include 2-4 conserved cysteines which form disulfide loops, a type II fibronectin-like domain and, except for the IL-7 and GH receptors, a conserved WSXWS motif (Cosman *et al.*, 1990). This motif is present as an analogous "aromatic-S-G-X-aromatic-S" sequence in the GHR. The length of the intracellular domain varies widely between family members, but a proline-rich region (Box 1) and, to a lesser extent, a charge-conserved region (Box 2) are commonly found proximal to the transmembrane domain (Sakamaki *et al.*, 1992; Colosi *et al.*, 1993; O'Neal & Yu-Lee, 1993). These regions are thought to be involved in signal transduction, and this structural conservation is also indicative of common signal transduction pathways (Fukunaga *et al.*, 1991; Colosi *et al.*, 1993). Indeed, the intracellular domains of family members may be interchangeable, as GH can stimulate proliferation via a GHR/G-CSF chimera containing the GH-binding domain and the G-CSF intracellular domain (Fukunaga *et al.*, 1991).

B. **The GH Receptor Gene**

The GHR is encoded by a single gene localized on chromosome 5.13 to p14 or 5p13 to p.12 in humans (Barton *et al.*, 1989; Arden *et al.*, 1990) but on the sex chromosome (chromosome Z) in chickens (Burnside *et al.*, 1991). This gene is composed of 10 exons ranging in size from 66 bp to 3400 bp stretching over a distance of >87 kb (Godowski *et al.*, 1989)(Figure 1.4). Exon 2 encodes the signal peptide, exons 3-7 encode for the extracellular domain, exon 8 encodes the transmembrane domain, and exons 9 and 10 encode for the intracellular domain of the receptor (Godowski *et al.*, 1989). Exon 1, which is not translated, consists of a series of alternatively spliced exons that encode the promoter. The promoter region of the ovine hepatic GHR mRNA has recently been cloned (O'Mahoney *et al.*, 1994). In addition to the TATA box and other components of the transcriptional machinery, response elements for a number of liver-specific transcription factors, for glucocorticoid and GH receptors and for R/EBP transcription factors have also been detected within this sequence (O'Mahoney *et al.*, 1994).

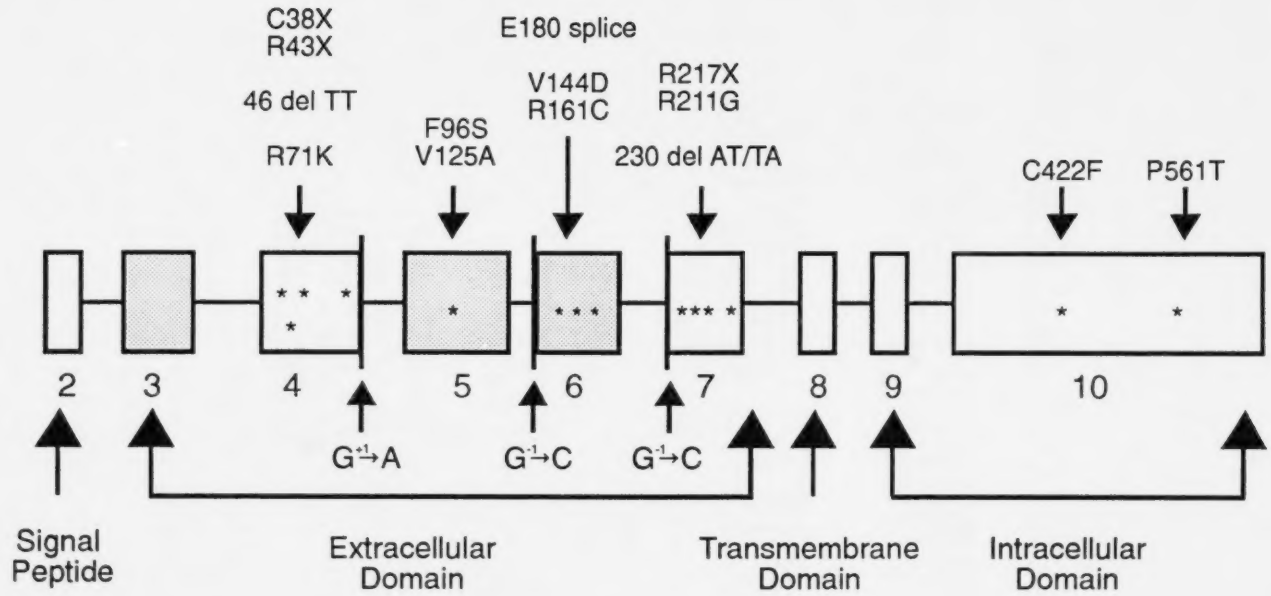

Figure 1.4. Structure of the GHR gene and mutations in Laron syndrome. The coding exons (boxes) are shown to approximate relative size, intron sizes are not to scale, and the non-coding part of exon 10 has been omitted. The structural protein domains encoded by the exons are indicated. The three shaded exons are involved in a deletion mutation. The location (asterisks) and types of known point mutations within exons 4-7 are summarized above the diagram. The three splice site mutations are indicated by black bars at the exon 4/intron 4, intron 5/exon 6 and intron 6/exon 7 boundaries and are described below the diagram. Based on Berg *et al.* 1992.

The GHR cDNA has been cloned in sheep (Adams *et al.*, 1990), humans (Leung *et al.*, 1987), rabbits (Leung *et al.*, 1987), mice (Smith *et al.*, 1988), bovines (Hauser *et al.*, 1990), pigs (Bingham *et al.*, 1991; Wang *et al.*, 1993) and chickens (Burnside *et al.*, 1991) and appears as a 4.2-4.7 kb mRNA transcript in Northern Blots. The biological relevance of several cloned GHR cDNA's has been demonstrated by transfection studies. In experiments using GHR-deficient cells or rat insulinoma cells, expression (or over-expression) of the full-length GHR in various cell lines dramatically increases their responsiveness to GH in stimulating lipolysis and lipogenesis, insulin and protein synthesis, and MAP kinase activation (Billestrup *et al.*, 1990; Emtner *et al.*, 1990; Fiddes *et al.*, 1992; Moller *et al.*, 1994). In addition to this full-length transcript, smaller transcription products of the GHR gene are also present in species other than humans, pigs, sheep and rabbits (Leung *et al.*, 1987; Smith *et al.*, 1988; Baumbach *et al.*, 1989; Bingham *et al.*, 1994). These transcriptional variants of the GHR mRNA are described below.

C. The GH Receptor Protein

i. Protein Size: The human and rabbit GHR is a single-chain polypeptide of approximately 638 amino acids (aa), composed of an 18 aa signal peptide, a 247 aa extracellular domain, a 24 aa transmembrane domain and a 349 aa intracellular domain (Leung *et al.*, 1987). The GHR in ruminants is highly similar (Adams *et al.*, 1990; Hauser *et al.*, 1990) whereas the chicken GHR is slightly smaller, largely due to the omission of exon 3 (Burnside *et al.*, 1991). This mammalian amino acid sequence predicts a protein of 70 kDa; however, GH/GHR complexes of 122-142 kDa, 200 kDa and/or 300 kDa have been purified from various species and tissues (Leung *et al.*, 1987; Yamada *et al.*, 1987; Hughes & Simpson, 1983; Fiddes *et al.*, 1992)(Table 1.2). The 120 kDa complex is probably the native form, corresponding to a 100 kDa protein bound to a single GH molecule (Yamada *et al.*, 1987). The larger forms may represent receptor dimers and trimers linked by disulfide bonds, as they are not observed in the presence of reductant (Table 1.2).

Table 1.2: Identified molecular weights of GH/GHR complexes under reducing and non-reducing conditions.

Receptor Source	Native Mr (kDa)	Reduced Mr (kDa)
rat adipocyte	56	56
	108	↑ 108
	240	↓ 230
	310	↓ 310
rat liver	130	100
	220	
	300	
rat liver	66	66
rat hepatocyte	100	↑ 100
	220	↓ 220
	300	↓ 300
rabbit mammary	61	61
rabbit liver	70-80	
rabbit liver	56	
	68	
	76	
rabbit liver	57	57
	124	124
mouse liver		56
		62
		125
transgenic mouse liver		70
		82
		122
human IM-9	140	140
	270	
human IM-9	130	130

Table 1.2. cont.

Receptor Source	Native Mr (kDa)	Reduced Mr (kDa)
human fibroblast	140	
human liver	124	↓ 124
	75	↑ 75
pig adipose	114	
	75	
sheep GHR cDNA (expressed in CHO)	170	170
	95	95
sheep liver		155
		80
pig liver	140	
chicken liver and pituitary	80-86	

The observed size of the GHR (100 kDa) is approximately 30 kDa larger than that predicted by its amino acid structure. This discrepancy may be due to post-translational modifications, such as glycosylation and covalent linkage to ubiquitin (Leung *et al.*, 1987). GH/GHR complexes containing a GHR that is equivalent to or smaller than the computed Mr have also been isolated (Hughes & Simpson, 1983), but these studies may be detecting the 55 kDa truncated GHBP, GHR degradation products, or the 50-60 kDa prolactin receptor (Borst & Sayare, 1982).

ii. The Intracellular Domain: The intracellular domain of the GHR is thought to mediate signal transduction, and may be further sub-divided into subdomains responsible for distinct GH actions. For example, the distal half is neccesary for GH induction of Serine protease inhibitor 2.1 (Spi2.1) and SRIF transcripts and for GH-induced insulin synthesis whereas the proximal portion may mediate GH-induced lipogenesis, c-fos and microtubule-associated protein (MAP) kinase activation and internalization (Moldrup *et al.*, 1991; Billestrup *et al.*, 1993; Enberg *et al.*, 1994; Gong *et al.*, 1994; Moller *et al.*, 1994). The prolines in this proximal region appear to play an especially important role, as proline-to-alanine mutations eliminate the ability of GH to stimulate receptor internalization, c-fos transcription, and MAP kinase activity (Billestrup *et al.*, 1993).

iii. The Extracellular Domain: The extracellular domain of the human GHR/GHBP, which is responsible for ligand binding, is composed of 2 immunoglobin-like domains separated by a 4-residue connecting chain (DeVos *et al.*, 1991). The 2 domains are linked by a salt bridge (DeVos *et al.*, 1991), and 3 disulfide loops between conserved cysteines in these domains also contribute to tertiary structure (Fuh *et al.*, 1990). One of these loops interacts with GH via electrostatic and hydrophobic interactions, and mutations within the loop eliminate GH binding in rabbits (Gobius *et al.*, 1992). Additional residues important for hGH binding are largely concentrated in the NH_2, disulfide-rich domain (Bass *et al.*, 1991). The extracellular domain of the GHR/GHBP is also subjected to numerous post-translational modifications, including covalent linkage to ubiquitin (Leung *et al.*, 1987) and N-glycosylation at asparagine residues (Asakawa *et al.*, 1986; Leung *et al.*, 1987).

Glycosylation state does not, however, affect ligand binding, as unglycosylated recombinant GHBPs are capable of binding GH with equal affinity (Fuh *et al.*, 1990). The relevance of ubiquitin linkage to GHR function is also unclear.

iv. **Mechanism of GH Binding:** Although early binding studies assumed 1:2 stoichiometry in GH/GHR complexes, recent work has demonstrated that one hGH molecule binds two hGHBP molecules and probably two GHR molecules (Ultsch *et al.*, 1991). The two binding sites on the growth hormone are distinct, but the residues interacting with the two sites differ in only one residue (Asn-19)(Cunningham *et al.*, 1991; Demeyts, 1992). Binding occurs sequentially to site I, which has higher affinity, and then to site II, and the resulting complex is stabilised by covalent and eventually disulfide interactions between the COOH tails of the two receptors (Frank *et al.*, 1994). This interaction between the COOH tails is also thought to initiate signal transduction and GHR internalization. Inhibition of dimerization occurs in response to GH analogs containing only one binding site, mutated GHBPs or GHBP antibodies and results in a blockage of GH action (DeVos *et al.*, 1991; Wells *et al.*, 1993; Ilondo *et al.*, 1994a; Ilondo *et al.*, 1994b). GHR/GHBP dimerization is also inhibited by high concentrations of GH; thus monomeric GH:GHBP complexes predominate in plasma (Wells *et al.*, 1993; Baumann *et al.*, 1994)(Figure 1.5). However, at the cell membrane, GHR concentrations greatly exceed GH concentrations and thus dimeric complexes are formed (Baumann *et al.*, 1994). The importance of dimerization in GH action may therefore explain the bell-shaped dose-response curve for GH in proliferative assays (Fuh *et al.*, 1992).

The specificity and magnitude of GH binding are dependent upon temperature, pH and the presence of divalent and monovalent cations and vary somewhat between species and between tissues (Table 1.3)(Barnard & Waters, 1988; Gray *et al.*, 1990). Calcium is necessary for the binding of hGH (but not oGH, rGH or bGH) to GHBPs and/or GHRs (Fix *et al.*, 1981; Barnard & Waters, 1988), as it negates opposed negative charges found in the hGH (but not in oGH, rGH or bGH) and the GHR. The inclusion of calcium increases the affinity of the GHBP for hGH by providing additional ionic bonds. This hGH:calcium:GHBP interaction may thus account for the increased affinity of hGH in

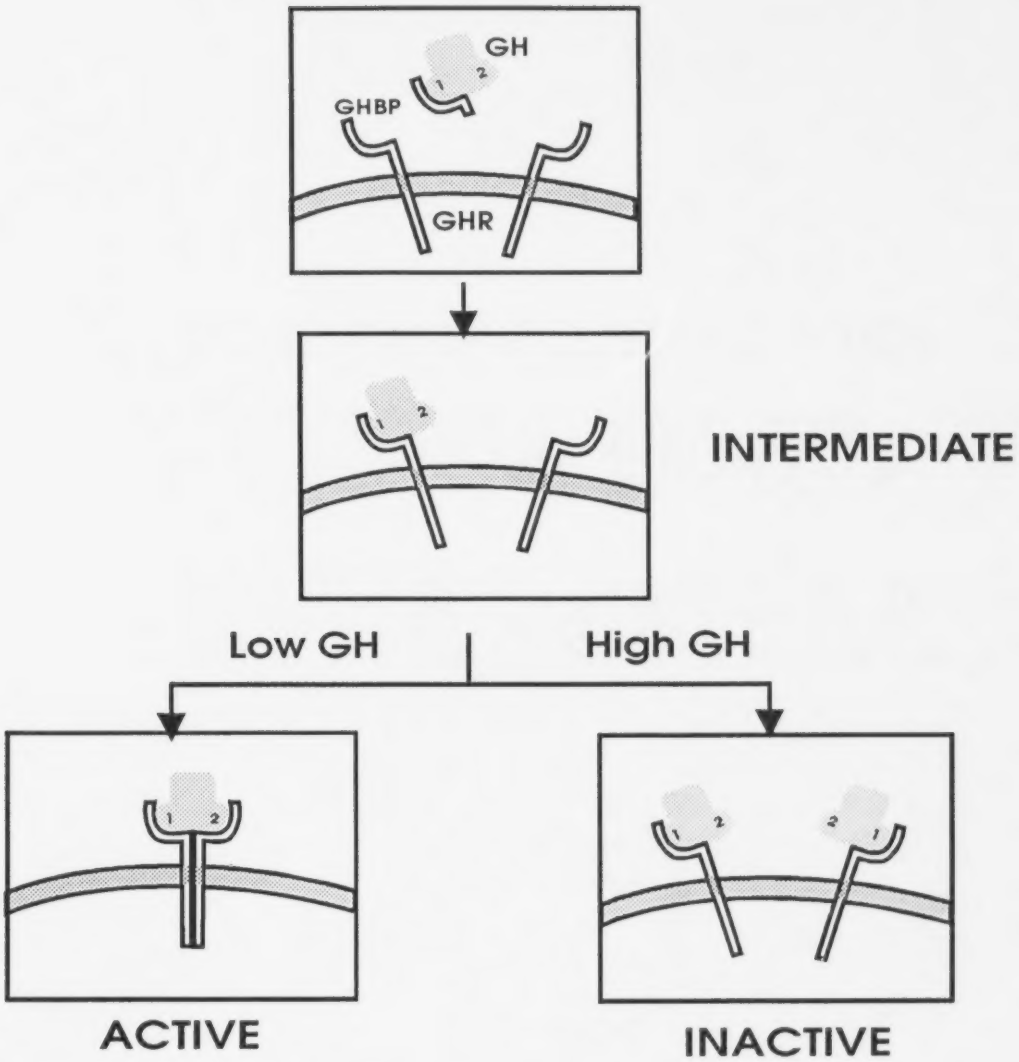

Figure 1.5: Sequential dimerization model for activation of the GH receptor. GH in plasma is bound to a single GHBP molecule at site 1. GH will dissociate from GHBPs and bind to tissue GHRs due to the higher affinity or abundance of tissue GHRs. At low GH concentrations, GH binds first at site 1 and subsequently at site 2 to produce an active GH:$(GHBP)_2$ complex. At high concentrations, GH saturates the GHR at site one and acts as an antagonist. Based on Wells *et al.* 1993.

Table 1.3: The affinities and capacities of growth hormone receptors from various species and tissues. The Bmax is described as fmol/mg protein unless otherwise described.

SPECIES	TISSUE	LIGAND	Affinity	Bmax
TILAPIA	liver	bGH	Kd = 13 nM	180
		tGH	Kd = 2.5 nM	390
EEL	liver	eGH	Ka = 1.1 nM^{-1}	105
SALMON	liver	sGH	Ka = 92 nM^{-1}	84
TROUT	liver	sGH	Ka =2.4 nM^{-1}	187 pmol/mg tissue
RAT	adipocyte	rGH	Ka = 1 nM^{-1}	15000/cell
(8.d ♂ or♀)	liver	rGH	Ka = 0.66 nM^{-1}	6.4
(28d. ♂)	liver	rGH	Ka = 0.66 nM^{-1}	30
(28d.♀)	liver	rGH	Ka = 0.66 nM^{-1}	39
(6 mo. ♂)	liver	oGH	Ka = 8.56 nM^{-1}	26.2
			Ka = 0.25 nM^{-1}	253
(20 mo. ♂)	liver	oGH	Ka = 9.98 nM^{-1}	253
			Ka =0.3 nM^{-1}	454
(adult ♀)	liver	bGH	Ka = 0.8 nM^{-1}	35 pmol/mg tissue
MOUSE	liver	bGH	Ka = 23.2 nM^{-1}	
GUINEA PIG	liver	oGH	Kd = 2.34 pmol/l	623
HUMAN	liver	hGH	Ka = 2.0 nM^{-1}	14-53
			Ka =1.5 nM^{-1}	707
	adipocyte	hGH	Ka = 2.1 nM^{-1}	7.3/cell
	lymphocyte	hGH	Ka = 1.5 nM^{-1}	7.1/cell
RABBIT	liver	bGH	Ka = 3 nM^{-1}	
	liver	rGH	Ka =0.17 nM^{-1}	19-68
			Ka = 10 nM^{-1}	173-802

Table 1.3, cont.

SPECIES	TISSUE	LIGAND	Affinity	Bmax
		rbGH	Ka =0.14 nM^{-1}	7-38
			Ka = 14 nM^{-1}	990-480
	♂ liver	hGH	Ka = 3.64 nM^{-1}	355
(adult)	lung	hGH	Ka = 2.6 nM^{-1}	9.5
(fetus)	lung	hGH	Ka =1.85 nM^{-1}	27.6
PIG	muscle	bGH	Ka = 9-15 nM^{-1}	5-6
(2.d)	liver	bGH	Ka = 3.5 nM^{-1}	12
(adult)	liver	bGH	Ka = 5.0 nM^{-1}	91
			Ka =0.35 nM^{-1}	
	liver	hGH	Ka = 1 nM^{-1}	
SHEEP	liver	oGH	Ka = 0.27nM^{-1}	
	expressed	oGH	Ka = 0.3 nM^{-1}	
		oPL	Ka = 0.76 nM^{-1}	
CHICKEN	liver	cGH	Ka =0.12 nM^{-1}	1.91
	liver	cGH	Ka = 8.4 nM^{-1}	11.23
	hypothal.	cGH	Kd=33.5 nM	2.14
TURKEY	liver		Ka =3.09 nM^{-1}	2.13

comparison to oGH or rGH in homologous and heterologous binding assays. Scatchard analysis generally results in a linear graph, and indicates the presence of a single class of binding sites, both high- and low-affinity binding sites have been identified in most species (Hughes, 1979; Baxter *et al.*, 1980; Hocquette *et al.*, 1989).

D. GH Receptor Heterogeneity

Although only one GHR/GHBP gene is present in the genome, GH-binding activity resides in a family of related proteins. This is thought to result from variations in transcription and post-translational processing of the GHR.

i. Transcriptional Variation: The best-characterised transcriptional variant of the GHR is the 1.2 kb transcript observed in rodents, which results from alternate splicing at the exon 7/8 boundary and contains a short hydrophilic tail in place of the transmembrane and intracellular domains. Translation of this transcript in rats results in 44 kDa and 52 kDa serum GHBPs (Frick *et al.*, 1994). This variant also encodes a short (38-42 kDa), unsecreted form of the GHR (GHRsh) in adipocytes that binds GH with high-affinity and is regulated independently of the long form (Frick & Goodman, 1992). The size difference between the GHRsh and the GHBP is due to differential glycosylation, which may facilitate GHBP secretion (Frick *et al.*, 1994).

Additional sites of alternative splicing include the exon3/exon4 junction, which results in GHRs with (GHR) or without (GHR3d) exon 3(Sobrier *et al.*, 1993; Mercado *et al.*, 1994). Splice site selection is regulated, as the 2 isoforms are present in different ratios in different human tissues. However, the functional significance of GHR3d is unknown, as GHR and GHR3d do not differ in affinity for 22 kDa or 20 kDa hGH or for the placental GH variant (hGH-V) (Urbanek *et al.*, 1992; Mercado *et al.*, 1994). The use of different splice sites at the exon1/exon 2 junction is also regulated in a tissue-specific manner, resulting in variation in the promoter-containing 5' untranslated region (Adams *et al.*, 1990; Pekhletsky *et al.*, 1992). Although variation in exon 1 would not result in structural differences, the use of alternative 5' non-coding exons may determine tissue- and perhaps developmentally-specific patterns of GHBP and GHR mRNA synthesis

(Bikbulatova *et al.*, 1994).

Variation in the 3'-untranslated region and in the selection of polyadenylation sites can also result in transcriptional heterogeneity. For example, the 2.6 kb transcript detected in rat liver mRNA is thought to result from excessive polyadenylation or alternative processing of the 3'-untranslated region of the rat 1.2 kb GHBP mRNA (Tiong & Herington, 1991a). The 2.6 kb transcript detected in ovine and chicken liver RNA may arise from a similar mechanism (Klempt *et al.*, 1993; Oldham *et al.*, 1993). Conversely, selection of an alternate polyadenylation site in exon 5 of the chicken GHR mRNA results in a 0.7 kb variant (Oldham *et al.*, 1993). Although this variant does not encode the characterized chicken GHBP (Oldham *et al.*, 1993), its close association with polyribosomes suggests translation into an unidentified protein.

ii. **Protein Variants:** Variation can also occur in proteins translated from the same mRNA due to differential post-translational processing and/or association with other proteins. For example, GHR sub-classes can be detected in rabbit liver and adipose by their reactivity to various monoclonal antibodies (Barnard *et al.*, 1990). The tissue-specific distribution of these sub-classes suggests that their immunological differences may be caused by interactions with tissue-specific membrane proteins. GHRs also differ in their affinity for homologous (Barnard *et al.*, 1988) and heterologous (Hughes *et al.*, 1983) GH, for cleaved GH (Ingram *et al.*, 1992) and for the 20K GH variant (Hughes *et al.*, 1983), and these variants can be differentially regulated (Hughes *et al.*, 1983).

E. Receptor Biosynthesis and Turnover

The biosynthetic pathway of the GHR is common to most peptides, in that the protein is synthesized on the rough endoplasmic reticulum, processed and glycosylated by the Golgi Apparatus and subsequently inserted into the plasma membrane. GHR synthesis is extremely rapid, as adipocytes require only two hours to restore GH-binding levels to normal following trypsinization (Gorin & Goodman, 1985; Roupas & Herington, 1988). The accelerated rate of GHR synthesis is necessitated by the rapid turnover of GHRs, as the receptor half-life in adipocytes, hepatocytes and fibroblasts varies between 40 and 75

minutes (Murphy & Lazarus, 1984; Gorin & Goodman, 1985; Amit *et al.*, 1993b). This high turnover rate is due to internalization by both constitutive and ligand-induced pathways (Roupas & Herington, 1988; Roupas & Herington, 1989). In the ligand-induced pathway, the GH-GHR complexes are endocytosed and processed through prelysosomal and lysosomal components, in which they are degraded (Roupas & Herington, 1988; Roupas & Herington, 1989). However, the ability of GH and its receptor to avoid lysosomal degradation is shown by the release of internalized, intact GH from IM-9 cells (Ilondo *et al.*, 1992) and by the reappearance of internalized GHRs in the plasma membrane (Roupas & Herington, 1988).

The rapid turnover and internalization of the GHR are thought to enable recognition of specific GH pulses and to permit responses based on the frequency of GH pulses (Bick *et al.*, 1989b; Bick *et al.*, 1989c). In the normal male rat, GH is secreted in a pulsatile fashion with a three-hour interpulse interval. Both somatogenic and lactogenic receptors are present at maximal levels during and immediately after a GH pulse, thus maximizing sensitivity (Bick *et al.*, 1992). During the trough period, GHRs are largely confined to the Golgi fraction due to increased ligand-induced internalization, and GHR recycling and biosynthesis that restore membrane GHR levels before the next GH pulse (Bick *et al.*, 1992).

The internalization of GH/GHR complexes ensures that the interaction between GH and the GHR does not demonstrate simple equilibrium kinetics. Instead, there is a time-dependent increase in apparently irreversible binding (Rosenfeld & Hintz, 1980). Thus, Scatchard analysis is less applicable for the GHR than for other receptors, but still provides an indication of the relative affinity and capacity of the GHR in different species and tissues.

F. GH Receptor Localization

Growth hormone receptors are ubiquitously present in rodents (rat and mice), ruminants (sheep and bovines), rabbits, humans, fish, and birds (chickens) (Table 1.4). Growth hormone binding-activity has also been localized in undomesticated mammals (Ng *et al.*, 1993), reptiles (Marques *et al.*, 1979), and amphibians (Posner *et al.*, 1974). In

most species, the adult liver is the primary GH target site, as GHR mRNA is 3- to 10-fold more abundant in liver than in other tissues (Scott *et al.*, 1992; Walker *et al.*, 1992). However, GH-binding and/or GHR mRNA are higher in frog kidney (Posner *et al.*, 1974) and fetal calf thymus (Scott *et al.*, 1992) than in liver, suggesting that the variable roles of GH in different species and ontogenic states may be mediated in part by altered GHR levels. Tissue specificity is also observed in the ratio between the 4.4 kb and 1.2 kb transcripts in rats, as it varies between 7.1 in liver to 0.3 in spleen (Bingham *et al.*, 1994). These ratios also vary during ontogeny and may be altered by physiological stimuli (Walker *et al.*, 1992; Bingham *et al.*, 1994). This differential production of the GHBP and the full-length GHR can provide further insight into the role of GH and GH-binding activity in different tissues.

Despite the ubiquitous nature of the GHR in most species, the intratissue localization of the GHR is often quite specific and, when compared to the distribution of the IGF-I receptor, delineates spheres under direct and indirect (IGF-I-mediated) GH influence. This strategy has been used in the kidney to identify the proximal tubule as a GH-responsive structure and the glomerulus as a IGF-I-responsive structure (Chin *et al.*, 1992). In addition, GHR expression is often limited to specific developmental stages which are responsive to GH action. For instance, the closure of the epiphyseal growth plate in rabbits is associated with the loss of GHRs, suggesting that GH is primarily involved in the differentiation of prechondrocytes into mature chondrocytes rather than in bone maintenance (Barnard *et al.*, 1988).

The intracellular distribution of GHRs and/or GHBPs is equally widespread, as GHR/GHBP immunoreactivity and binding activity have been localized to plasma membranes, secretory vesicles, Golgi Apparatus and nuclei in numerous cell types (Lobie *et al.*, 1990; Fraser & Harvey, 1992; Hull *et al.*, 1992; Lobie *et al.*, 1992). Possible roles for these intracellular GH-binding proteins are discussed in section 1.3.

Table 1.4. Distribution of GHR mRNA and immunoreactive and/or bioactive GHRs. (Based on Harvey and Hull 1994b).

TISSUE	HUMANS		RODENTS		RUMINANTS		FISH		BIRDS	
	GHR mRNA	GHR	GHR mRNA	GHR	GHR mRNA	GHR	GHR mRNA	GHR	GHR mRNA	GHR
liver	low*		+,+*	+,low *	+, low*	+		+	+	
kidney		+*	+,+*	+*	+,low*			+,-	+	
adrenal	+*	-*	+	+*	+				+	
heart	+*		+		+			-	+	
lung	+*	-*	+,+*	+*	+, low				+	
skin	+	+,+*		+*,+				+,-		
fibroblasts	+*			+*						
muscle	+*	-*	+	+*	+,+*			+,-	+	
chondro-cytes /bone	+*	-* +		+*						
GIT	+*	-*	+,+*	+*,+	+			+,-		
fat		+	+	+	+			+	+	
mammary gland			-		+					
thymus			-	+	+,*				+	

Table 1.4., cont.

TISSUE	HUMANS		RODENTS		RUMINANTS		FISH		BIRDS	
	GHR mRNA	GHR	GHR mRNA	GHR	GHR mRNA	GHR	GHR mRNA	GHR	GHR mRNA	GHR
spleen	+*		-		+*			+		
hemato-poietic cells			+							
Ant.Pit-uitary	+		+	+*	+				+	
Post.Pit-uitary					low					
brain	+*	+*	-,+		low,+*			+	+	
				+*				-		
tongue			+							
gall-bladder	-*									
pancreas	+*			+*	low					
urinary tract	+*			+						
male reprod. tract				+	*					

Table 1.4. cont.

TISSUE	HUMANS		RODENTS		RUMINANTS		FISH		BIRDS	
	GHR mRNA	GHR	GHR mRNA	GHR	GHR mRNA	GHR	GHR mRNA	GHR	GHR mRNA	GHR
ovary	+*		+	+	+			+		
granulosa	+	+	+	+						
corpus lutea			+	+	+					
testis	-*		-	+	+			+	+	
endometrium				+						
placenta	+	+			low,+					
teeth				+						
gill								+, -		

+ denotes the presence of GHR mRNA or protein
- denotes the absence of GHR mRNA or protein
* denotes studies perfomed in fetal animals

Rodents: rats and mice
Ruminants: sheep and bovines
Birds: chickens

1.3 GROWTH HORMONE BINDING PROTEINS

Growth hormone is a hydrophilic protein and hence soluble in blood plasma. Nevertheless, 30-50% of circulating GH is bound to a heterogenous group of saturable plasma proteins of different molecular size, structure, abundance, and affinity (reviewed in Herington *et al.*, 1986a; Baumann, 1991a; Baumann, 1993; Kelly *et al.*, 1993; Harvey & Hull, 1994).

A. Characterization

i. GHBP Structure: GHBPs of varying size (eg. 61 kDa in man , 42 kDa in mice (Smith & Talamantes, 1988); 39 and 45 kDa in rats (Sadeghi *et al.*, 1990); 50-60 kDa in pigs (Davis *et al.*, 1992); 50-60 kDa in chickens (Vasilatos-Younken *et al.*, 1991)) have been identified in animal sera that differ between species in structural and immunological relatedness (Spencer *et al.*, 1988; Baumann & Shaw, 1988; Barnard *et al.*, 1986). The binding of GH in each species may also be to heterogenous plasma components, since proteins with differing binding affinities, molecular mass and amount of glycosylation have been detected in rat sera (Smith *et al.*, 1989; Haldosen & Gustafson, 1990; Massa *et al.*, 1993; Baumann, 1991a). Most of these components do, however, appear to be related to the membrane-bound liver GHR (Leung *et al.*, 1987; Spencer *et al.*, 1990). The GHBP in rat and mouse, for example, is identical to the extracellular domain of the liver membrane GHR, although a short C-terminal hydrophilic tails of 17 and 25 amino acids (respectively) are substituted for the transmembrane and intracellular domains of the GHR (Smith & Talamantes, 1987; Baumbach *et al.*, 1989). This hydrophilic tail ensures the secretion of the GHBP in a soluble form. The high-affinity GHBP in humans and rabbits is also identical to the extracellular domain of their GHRs, but is not believed to contain the hydrophilic tail (Barnard & Waters, 1985; Baumann & Shaw, 1988).

ii. GHBP Binding: In man, most (85-90%) of the protein-bound 22 kDa GH monomer in plasma is bound to the high-affinity, (Ka = 3.9 x 10-8 M) low capacity (approximately 0.5-2.0 nmol/l) GHBP described above (Baumann *et al.*, 1990). This GHBP binds the

placental GH variant (GH-V) with high-affinity and the 20 kDa GH variant with 20-fold less affinity but does not bind non-human GH, human prolactin or placental lactogen (Baumann *et al.*, 1986; Baumann *et al.*, 1991; Baumann, 1991a; Blumenfeld *et al.*, 1992). The formation in plasma of GH complexes of 80-85 kDa suggests a 1:1 binding stoichiometry for the GH:GHBP interaction (Wells & DeVos, 1993; Wells *et al.*, 1993).

Although guinea pigs appear to lack plasma GHBPs (Amit *et al.*, 1992b; Fairhall *et al.*, 1992), binding proteins for GH are also present in the plasma of rats, mice, rabbits, cats, dogs, pigs, goats, sheep, cattle, horses and monkeys. These may reflect four classes of binding proteins, on the basis of differences in binding specificity (Amit *et al.*, 1992b). Type 1 GHBPs, found in rat and mouse serum, have a higher affinity for somatogenic (bGH, rGH) GH preparations than for lactogenic hormones (hGH, prolactin). Type II GHBPs are found in the goat, sheep and cow and have low affinity for hGH but an even lower affinity for bGH, rGH or prolactin (Davis *et al.*, 1992). The type III GHBP consist of a mixed GH/prolactin binding site and is found in the rabbit, horse, dog, cat and pig, whereas the type IV GHBP is human-like and found in the monkey. The affinity of hGH binding is highest in type III species, followed by type IV and type II. Amit *et al.* (1992b) also claims that binding capacities of these plasma proteins are greatest in the rabbit (a type III animal) and least in type 0 animals (which lack circulating GHBPs) and type 1 animals. However, in the study of Bingham *et al.* (1994), GH-binding activity was significantly higher in rat serum (type 1) than in pig serum (type III) and bovine serum was devoid of detectable GH-binding activity.

A second GHBP, which is specific for hGH, is detectable only in human plasma (Baumann & Shaw, 1990; Baumann, 1991a; Baumann, 1991b; Baumann, 1993). However, in contrast with the high-affinity GHBP, this 100 kDa protein or family of proteins is structurally and immunologically unrelated to the membrane GHR or the high-affinity GHBP (Baumann *et al.*, 1987b; Baumann & Shaw, 1988; Baumann & Shaw, 1990). It has low affinity (approximately 10-5 M) but high binding capacity for 22 kDa hGH (Baumann & Shaw, 1990). Unlike the high affinity GHBP, this protein also binds 20 kDa GH with equal or higher affinity than 22 kDa hGH (Baumann & Shaw, 1990) and complexes the majority of protein-bound 20 kDa hGH (Baumann *et al.*, 1989a). This

specific binding of 20 kDa GH may be to a specific binding site on the low-affinity GHBP or to a separate specific low-affinity GHBP (Baumann & Shaw, 1990; Baumann, 1991a; Baumann, 1993).

The low affinity GHBP is thought to be produced in the liver, although its plasma concentration appears to be differentially regulated (Baumann *et al.*, 1989c; Tar *et al.*, 1990) from the high-affinity GHBP. For instance, low-affinity binding is increased in patients with liver disease, renal failure or acromegaly (Baumann *et al.*, 1989a). Changes in the high- and low-affinity GHBP are similarly discordant during ontogeny (Merimee *et al.*, 1992) and pregnancy (Merimee *et al.*, 1992) and in relation to gonadal and somatotrophic status (Postel-Vinay *et al.*, 1991b). Moreover, while high-affinity GHBP levels are independent of gender, low-affinity binding sites are more numerous in women than in men (Baumann *et al.*, 1989a).

B. GHBP Synthesis and Release

The rodent GHBP is probably produced entirely from translation of the truncated (1.2 kb) GHBP transcript (Sadeghi *et al.*, 1990; Spencer *et al.*, 1990)(Figure 1.6). The absence of GHBP in the media of COS-7 cells transfected with the full-length rat GH receptor cDNA (Sotiropoulos *et al.*, 1993) supports this view, especially as the mouse GHBP is abundantly present in the media of COS-7 cells transfected with the 1.2 kb mouse GHBP cDNA (Cramer & Talamantes, 1993). A putative chicken GHBP (CH17) has also been identified from cDNA libraries that results from alternative splicing and contains the extracellular domain and at least 600 bp of the intracellular domain (Bingham *et al.*, 1994). However, translation of this cDNA has not yet been demonstrated *in vivo.*

In humans, rabbits, sheep and pigs the circulating GHBP may arise from the proteolytic clevage of membrane receptors, since only a single, full-length GHR transcript is present in their mRNA (Bingham *et al.*, 1994; Smith *et al.*, 1988; Leung *et al.*, 1987)(Figure 1.6). This hypothesis is also supported by the ability of cultured human IM-9 lymphocytes to 'shed' the extracellular portion of their receptors in response to sulfhydryl inactivating reagents, which may activate a protease that cleaves the receptor close to the extracellular domain (Trivedi & Daughaday, 1988). Moreover, high levels

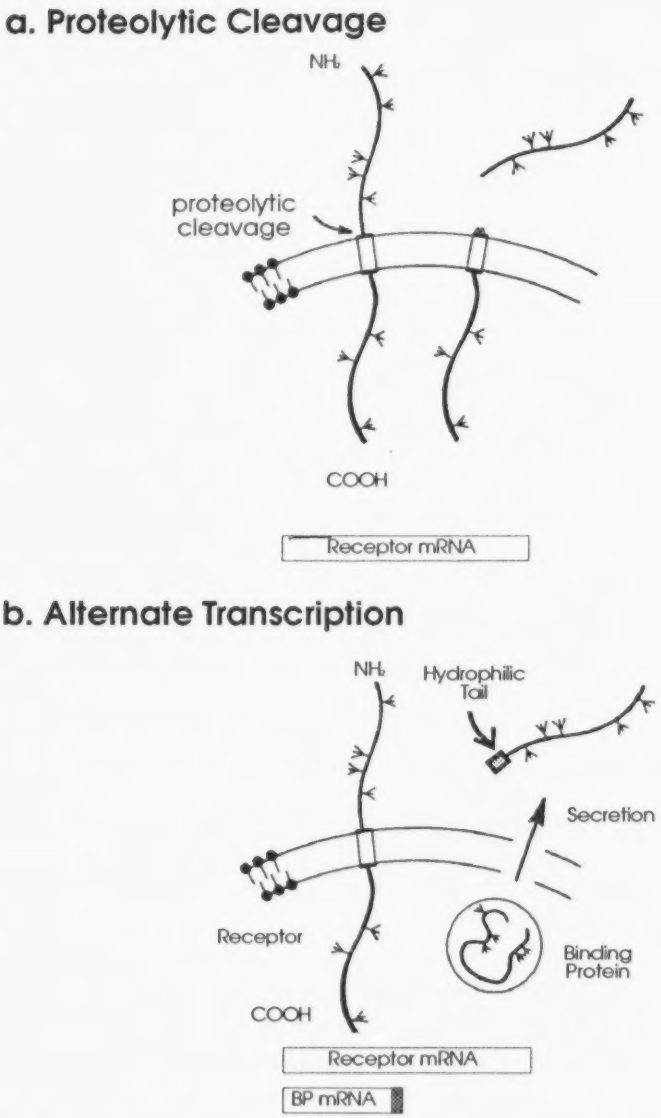

Figure 1.6. Two possible mechanisms for the generation of the high-affinity growth hormone (GH) binding protein. (a) Proteolytic cleavage of the GH receptor near the transmembrane domain and shedding of the binding protein into extracellular space. (b) Separate synthesis of receptor and binding protein, with secretion of the latter. In this case, separate mRNAs for receptor and BP are produced either from two different genes (not known to exist) or through two alternative mRNA splicing mechanisms (in rats and mice) that contain a BP-specific hydrophilic tail. Tree structures denote putative N-linked oligosaccharides. Based on Baumann, 1990.

of GHBP are secreted by CHO cells transfected with rabbit GH receptor cDNA, which only transcribes a 4.5 kb message for the full-length receptor (Sotiropoulos *et al.*, 1993). This proteolytic process is likely to occur at the cell surface and may be mediated by covalently-linked ubiquitin (Leung *et al.*, 1987) but may also occur proximal to insertion of the GHR in the plasma membrane. This possibility is supported by the accumulation of GHBP in the plasma of cyclohexamide-treated rats deficient in membrane GHRs, suggesting the mobilization of GHBPs from intracellular pools (Amit *et al.*, 1993b). Moreover, the total amount of binding protein in rabbit serum is far greater than that present in liver membranes (Ymer & Herington, 1985).

The tissue source of the circulating GHBP has not been conclusively identified, but the abundance of GHBP mRNA in the liver and the secretion of GHBP from cultured mouse hepatocytes (Barnard *et al.*, 1994) and a human hepatoma cell line (Amit *et al.*, 1994) suggests a hepatic site of origin. Although most tissues contain the 1.2 kb GHBP transcript and intracellular GHBPs, only certain tissues contribute to the circulating GHBP pool, as GHBPs are not observed in the media of cultured rat adipocytes (Frick & Goodman, 1992) and mouse trophoblasts (Barnard *et al.*, 1994).

C. TISSUE GHBPs

Although GHBPs were originally detected in serum, they are also abundantly present in tissues. GHBPs immunologically identical to the extracellular domain of the GHR are present in kidney, the gastrointestinal tract, in skeletal and muscular systems, in skin, in cardiovascular and respiratory systems and in central and peripheral nervous systems (Harvey & Hull, 1994; Harvey & Hull, 1994b, and references therein). In each tissue, immunoreactivity for the GHBP was both cytoplasmic and nuclear. GHBPs have also been identified on the basis of soluble GH-binding activity in rabbit liver and kidney cytosol (Barnard & Waters, 1985; Herington *et al.*, 1986b; Ymer *et al.*, 1989) and in cytoplasmic and nuclear fractions of the rat (Barnard & Waters, 1985; Fraser & Harvey, 1992) and human (Hocquette *et al.*, 1990) liver. The ubiquitous nature of tissue GHBPs and their localization in cells lacking ready access to serum (eg. neurons and oocytes, Lobie *et al.*, 1991; Lobie *et al.*, 1992; Lobie *et al.*, 1993) suggests that they may be

produced within individual tissues.

Tissue GHBPs vary from serum GHBPs and differ in size between tissues. For instance, a recent study by Lai et al. (1993) detected a 58 kDa GHBP in rat pituitary glands, but a 48 kDa GHBP in rat hippocampus. This variation may be due to differential glycosylation, as deglycosylation obliterates the size difference between the membrane-bound GHBP (GHRs) found in adipocyte membranes and the serum GHBP (Frick *et al.*, 1994).

D. GHBP Roles

i. Intravascular GHBPs: Circulating binding proteins may increase ligand solubility and hence ligand concentrations in plasma. However, as high-affinity GHBPs have the same pI as GH, increasing GH solubility is unlikely to be the role of plasma GHBP. Instead, the binding of GH to plasma proteins may have a 'buffering' capacity to dampen episodic variations in the amount of free (biologically active) hormone available to target sites (Veldhuis *et al.*, 1993a; Veldhuis *et al.*, 1993b), as GH release is highly pulsatile. The association of GH with plasma proteins is, at least in children, increased when free GH concentrations are elevated, whereas dissociation in increased when free GH concentrations are low (Hochberg *et al.*, 1991b; Carlsson *et al.*, 1993). This role of the GHBP is suggested by the inverse correlation between plasma GH pulsatility and GHBP concentrations, as GH pulsatility is usually low in animals with high GHBP levels (eg. female rats and rabbits) (Jansson *et al.*, 1985; Minamitani *et al.*, 1989; Amit *et al.*, 1992b; Carmignac *et al.*, 1992) and is high in comparatively GHBP-deficient animals (eg. male rats) (Jansson *et al.*, 1985; Amit *et al.*, 1992b). Thus, the GHBP may provide a GH reservoir to be mobilized during troughs in GH secretion, thereby ensuring that target tissues are exposed to relatively constant GH concentrations (Wells *et al.*, 1994). Alternatively, intravascular GHBPs could prevent low levels of GH from passing through the capillary barrier to target tissues, thereby raising the threshold concentration of GH required for the induction of biological activity and ensuring quiescent periods of GH activity between episodic pulses of GH release (Fairhall *et al.*, 1992).

The GHBP may also inhibit GH action in an autoregulatory fashion, since GH

increases GHBP concentrations (Robinson *et al.*, 1993) and the GHBP inhibits GH binding and action *in vitro* (Lim *et al.*, 1990; Mannor *et al.*, 1991; Dattani *et al.*, 1994). This inhibition may be due to the increased size of protein-complexed GH, which restricts GH to the vascular compartment and limits its access to target tissues (Amit *et al.*, 1992a). Moreover, the high-affinity GHBP may interfere with GH binding to the signal-mediating, full-length receptor by competitive inhibition, as the GHR and GHBP are generally similar in structure and GH affinity (Herington *et al.*, 1986b; Mannor *et al.*, 1991; Amit *et al.*, 1992a). However, the GHBP appears to play a stimulatory role in GH action *in vivo* by prolonging the half life of GH and thereby increasing GH availability (Baumann *et al.*, 1987a; Baumann *et al.*, 1989b). Indeed, the growth-promoting effects of GH in normal and dwarf rats and GH-deficient children are enhanced by co-administration of GHBP (Martha *et al.*, 1992; Robinson *et al.*, 1993). This potentiating action is due in part to decreased degradation of GH-GHBP complexes in the kidney, as the increased molecular size of the GH-GHBP complex prevents glomerular filtration (Baumann *et al.*, 1989b). Moreover, bound GH cannot interact with tissue receptors, thus diminishing receptor-mediated clearance in target cells (Roupas & Herington, 1989).

Actions of GH within specific tissues may also be modulated by binding proteins if the receptor and binding protein differ in affinity. For instance, the affinity of the hepatic GHR in mice is twenty-fold greater than the GHBP, and GH-GHBP complexes will dissociate in the presence of the GHR (Smith & Talamantes, 1988; Cramer *et al.*, 1992a). Since the amount of GHBP greatly exceeds the amount of GH in the circulation of pregnant mice, the binding protein may serve to direct GH to the liver and other tissues with pregnancy-induced increases in GHR expression (Cramer *et al.*, 1992a; Cramer *et al.*, 1992b; Cramer & Talamantes, 1993). Alternatively, GH:GHBP complexes may chimerically dimerize with the long and/or short form of the membrane-bound GHR and thereby modulate GH signal activation. However, experimental evidence supporting this possibility has not been obtained.

ii. Intracellular GHBPs: As described, GHBPs are also present intracellularly and may modulate GH action or metabolism. For instance, GHBPs may serve to transport GH

to nuclear sites of action (described in section 1.6) or to intracellular compartments for recycling or degradation (Harvey & Hull, 1994a). Alternatively, the binding of GH to intracellular GHBPs rather than to intracellular GHRs may competitively inhibit receptor-mediated GH actions. It is also possible that GHBPs have other roles independent of ligand binding. They may possess their own receptors, as has been shown for sex-hormone binding globulin and corticosteroid binding globulin (Rosner, 1990), and may induce cellular responses independent of or in conjunction with GH.

iii. Extracellular GHBPs: The localization of GHBPs within extracellular spaces, in breast milk (Postel-Vinay *et al.*, 1991a) and in follicular fluid (Amit *et al.*, 1993a) is suggestive of further GHBP roles. For example, complexing of GH to the GHBP within the extravascular space or within breast or follicular tissue would prevent GH diffusion across the capillary barrier, thus maintaining high concentrations of GH around cells (Fairhall *et al.*, 1992). Moreover, the recent identification of a placental 26 kDa GH variant possessing a hydrophobic domain suggests that GHBPs may serve as a ligand for this membrane-bound GH (Liebhaber *et al.*, 1989).

1.4 REGULATION OF THE GH RECEPTOR/GH BINDING PROTEIN

Regulation of the GHR occurs at both transcriptional and translational levels. Variation in serum and hepatic GH-binding activity is observed throughout ontogeny and pregnancy of most species, however; the magnitude and characteristics of the changes are highly species-specific. These changes are mediated largely by other hormones, as GH, estrogen, thyroid hormones, insulin and adrenal steroids are all regulators of GHR/GHBP expression and/or translation.

A. Ontogenic Regulation

Hepatic GHR/GHBP transcripts and binding activity are present pre-natally in rats (Garcia-Aragon *et al.*, 1992), sheep (Klempt *et al.*, 1993), fish (Marti-Palanca & Perez-Sanchez, 1994) and rabbits (Ymer & Herington, 1992) and generally increase until adulthood. However, ontogenic changes in GHR/GHBP expression and translation are

regulated in a highly tissue-specific, species-specific and transcript-specific manner. For instance, GHR mRNA increases 2-fold more than GHBP mRNA in the rat kidney and lung, whereas both transcripts decrease in the rat ileum between 1 and 42 days of age (Walker *et al.*, 1992). These tissue-specific ontogenic variations in tissue GHR distribution often correspond with ontogenic changes in GH roles. For example, the concentration of GHR mRNA or GH-binding activity in the kidney and muscle of the fetal sheep and in the brain of larval fish suggests that GH may be involved in prenatal neural development and metabolism, whereas the abundant hepatic GHRs in post-natal animals are indicative of the post-natal growth-promoting effects of GH (Adams *et al.*, 1990). Translational variants of the GHR are also differentially regulated throughout ontogeny, as the high- but not the low-affinity hepatic binding site increases with age in steers (Breier *et al.*, 1988).

The serum GHBP also increases during ontogeny in rats, pigs, and sheep (Mulumba *et al.*, 1991; Ambler *et al.*, 1992; Davis *et al.*, 1992) but decreases between hatch and 5 weeks of age in chickens (Tobar-Dupres *et al.*, 1993). In humans, GHBP levels are low in newborns and neonates and progressively rise until the second or third decade, with the largest increase occurring during the first few years of life (Silbergeld *et al.*, 1989; Martha *et al.*, 1993). The pubertal growth spurt is not, however, accompanied by increased serum GHBP levels (Massa *et al.*, 1992; Martha *et al.*, 1993). An ontogenic decline in the elderly (men in particular) has been proposed, but remains controversial (Daughaday *et al.*, 1987; Baumann *et al.*, 1989a).

B. Sexual Dimorphism

Hepatic GHR mRNA and tissue and/or serum GH-binding levels are sexually dimorphic in rats (Herington, 1982) but not in rabbits (Kelly *et al.*, 1974), mice (Sasaki *et al.*, 1982), cows (Hauser *et al.*, 1990) chickens (Tobar-Dupres *et al.*, 1993) or turkeys (Vasilatos-Younken *et al.*, 1990). GHBP levels are only slightly higher in human females than in males, but the difference may become more pronounced with age (Baumann *et al.*, 1989a; Tar *et al.*, 1990). The sexual variation in rat GHBP levels is observed after puberty and is GH-dependent, resulting in significantly higher levels of GHR/GHBP mRNA,

serum GHBP and hepatic GH-binding sites in adult female rats (Carmignac *et al.*, 1993a). The pubertal rise in estrogen (Carmignac *et al.*, 1993a) and the development of sexually dimorphic patterns of GH secretion (Maiter *et al.*, 1988a) have been implicated in the dramatic upregulation of GH-binding activity in post-pubertal female rats. Hormonal regulation of the GHR is discussed in more detail below.

C. Pregnancy

The significant changes in GH levels during pregnancy are mirrored by striking alterations in GHR and/or GHBP concentrations. In rats, the GHR and the GHBP are differentially regulated, as the 1.2 kb transcript and plasma GHBP are increased at late pregnancy whereas the 4.4 kb transcript and the hepatic GHR remain constant (Tiong & Herington, 1991a; Tiong & Herington, 1991b; Travers *et al.*, 1993). A similar dichotomy is observed in the rat placenta, in which GHBP mRNA and GHBP (rather than GHR) immunoreactivity are abundantly present (Barnard *et al.*, 1994). Conversely, both GHR and GHBP mRNA and immunoreactivity are increased in the late-pregnant mouse liver; thus, differences in GHR/GHBP regulation exist between these closely allied rodent species (Sasaki *et al.*, 1982; Sanchez-Jimenez *et al.*, 1990). In humans, plasma GHBP levels may increase during early pregnancy and progressively decline from the end of the first trimester to prepregnant GHBP levels (Blumenfeld *et al.*, 1992; Merimee *et al.*, 1992). Maternal and fetal factors play a role in GHR/GHBP regulation, as studies in mice have demonstrated that the pregnancy-induced GHR/GHBP upregulation is correlated with the number of conceptuses and is dependent upon adequate GH concentrations, the foeto-placental unit and the maternal adrenal gland (Sasaki *et al.*, 1982; Sanchez-Jimenez *et al.*, 1990; Cramer *et al.*, 1992b). Glucocorticoids may also be contributing factors, as they upregulate GHR concentrations (see below) and are present at increased concentrations in pregnant rats (Moldrup *et al.*, 1993).

D. Hormonal Factors

1. Growth Hormone: Although autoregulation of the GHR/GHBP has been the focus of intense study for many years, the true nature of the GH-GHR regulatory interaction

remains unclear. However, some tentative species-specific and tissue-specific conclusions can be drawn.

In rats depleted of pituitary GH by hypophysectomy, GHR mRNA and hepatic GH-binding are reduced in females (Herington *et al.*, 1976) but unchanged (Mathews *et al.*, 1989) or increased (Messini *et al.*, 1985) in adult and juvenile males. This sexual dimorphism may relate to the higher basal levels of GH-binding in female rats, which in turn may be due to the continuous nature of GH secretion in females compared to the pulsatile nature of GH secretion in males. Indeed, continuous GH administration or chronic exposure to high GH levels upregulates GH-binding and GHR/GHBP expression (Bick *et al.*, 1991; Aguilar *et al.*, 1992; Carmignac *et al.*, 1993b) in intact and hypophysectomized rats of both sexes, whereas intermittent or "pulsatile" GH administration in the male pattern is only minimally effective (Maiter *et al.*, 1992). In contrast, GHR/GHR mRNA levels are down-regulated by a single GH injection (Maiter *et al.*, 1988b); thus, GH appears to chronically up-regulate and acutely down-regulate its own receptors in the rat liver. GH also maintains its own receptors but causes acute downregulation in other species (eg. eel liver, Mori *et al.*, 1992) and tissues (eg. rat chondrocytes, Nilsson *et al.*, 1990).

However, in some species and tissues, hypophysectomy increases (eg. chicken liver, Vanderpooten *et al.*, 1991) or has no effect (eg. rat adipose, Mathews *et al.*, 1989) and chronic GH decreases (eg. salmon liver, Gray *et al.*, 1992) GHR levels; thus, it is impossible to definitively describe autoregulatory control of the GHR. Moreover, GHR levels are not consistently correlated with serum GH concentrations. For instance, the correlation between GHR and GH levels in turkeys varies between positive, 0 and negative values over ontogeny (Bacon *et al.*, 1989; Vasilatos-Younken *et al.*, 1990).

GH may also be necessary to maintain serum GHBPs in rodents, as GHBP levels are low in GH-deficient dwarf or hypophysectomized rats but increased to normal by continuous GH infusion (Bick *et al.*, 1989a; Bick *et al.*, 1991; Bick *et al.*, 1992). The human GHBP may also be GH-dependent, as GHBP levels are low in some GH-deficient children and are restored by long-term GH replacement (Hochberg *et al.*, 1991c; Postel-Vinay *et al.*, 1991b; Zadic *et al.*, 1993). However, GHBP activity is not abnormal

in other patients with GH excess or deficiency syndromes (Baumann *et al.*, 1989a; Hochberg *et al.*, 1991c; Merimee *et al.*, 1991; Ho *et al.*, 1993); thus, the regulatory influence of GH on human GHBP is uncertain. In contrast with hepatic receptors, plasma GHBP levels are not affected by acute changes in exogenous or endogenous GH in rats (Carmignac *et al.*, 1992; Robinson *et al.*, 1993) or humans (Hochberg *et al.*, 1991b; Carlsson *et al.*, 1993).

ii. Thyroid Hormones: Thyroid status appears to be directly correlated with hepatic GHR expression in mammals. For example, T3, alone or in conjunction with bGH, upregulates GH-binding sites in hepatocytes isolated from dwarf mice (Fouchera-Peron *et al.*, 1981) and hGH binding sites in hypophysectomized rat adipocytes (Gause & Eden, 1985). Plasma GHBP levels are also directly correlated with thyroid status, as hyper- and hypothyroidism in humans are associated with increased and decreased GHBP activity, respectively (Amit *et al.*, 1991). Conversely, exogenous thyroid hormones down-regulate GH-binding activity in chicken hepatic membranes (Vanderpooten, 1992) but have no effect in fish (Gray *et al.*, 1992).

iii. Glucocorticoids and Glucose: Reductions in levels of glucocorticoids and/or glucose may mediate the depression of GHR levels observed in fasted rats. Both compounds are decreased in parallel with the loss of receptors in starved rats (Maes *et al.*, 1983) and in nutrient-depleted cultured hepatocytes, and glucose and glucocorticoid administration to cultured hepatocytes restore GHR levels to 70% of the level in freshly isolated hepatocytes (Niimi *et al.*, 1991). A stimulatory effect of glucocorticoids on GHR mRNA is also observed in newborn rat pancreatic islets and in RIN-5AH cells (Moldrup *et al.*, 1993) but not in rat adipocytes (Gause & Eden, 1985), thus indicating tissue specificity.

iv. Gonadal Hormones: The influence of gonadal hormones on GHR levels may be responsible for increased GHR expression during pregnancy and may contribute to the sexual dimorphism of GHR expression in rats. These interactions are suggested by the

GH-dependent, upregulatory effect of estradiol on GH-binding sites in rat (Herington *et al.*, 1976) and bovine (Breier *et al.*, 1988) liver and rat serum (Robinson *et al.*, 1993). Conversely, testosterone reduces hepatic GH-binding in rats (Bryson & Baxter, 1986) and serum GHBP in humans (Postel-Vinay *et al.*, 1991b).

1.5 GH RESISTANCE

Tissue resistance to GH action is observed in numerous clinical, physiological and pathophysiological situations and is often associated with deficient, dysfunctional or absent GH receptors.

A. Nutritional status

Growth hormone receptor levels generally correlate well with nutritional status, and GHR deficiency has been implicated in the poor growth, GH-resistance and low IGF-I levels associated with nutritional deprivation and fasting (Postel-Vinay *et al.*, 1982; Maes *et al.*, 1983; Bornfeldt *et al.*, 1989). Short-term (3-4 d) but not long-term fasting severely reduces GHR mRNA and/or somatogenic receptor levels in most rat and chicken tissues but not in rat kidney, and GHR levels are normally restored by refeeding (Postel-Vinay *et al.*, 1982; Bornfeldt *et al.*, 1989; Vanderpooten *et al.*, 1989). GHBP levels in rats, chickens, pigs and humans are also reduced by short-term fasting and restored by refeeding (Baumann *et al.*, 1988; Straus & Takemoto, 1990; Mulumba *et al.*, 1991; Tobar-Dupres *et al.*, 1993). Thus, in total nutritional deprivation, a GHR deficiency could account for the observed GH-resistance. However, despite a similar degree of GH resistance in protein- or calorically-deprived animals, GH binding levels are depressed in young animals (steers and rats) but are relatively normal in adult sheep or rats (Maes *et al.*, 1984; Breier *et al.*, 1988; Fliesen *et al.*, 1989; Triest *et al.*, 1991; Bass *et al.*, 1992). A post-receptor defect is thought to be responsible for the decreased IGF-I levels in adult protein-restricted animals, as exogenous GH is unable to restore IGF-I concentrations despite normal hepatic GH-binding sites (Thissen *et al.*, 1990).

B. Pathophysiological GH Resistance

Hepatic and renal dysfunction in rats and humans are also associated with GH resistance and characterised by GHR-deficiency (Tonshoff *et al.*, 1990; Chang *et al.*, 1989; Hattori *et al.*, 1992; Tonshoff *et al.*, 1994). Curiously, experimental renal insufficiency in rats caused by partial nephrectomy results in a reduction in hepatic GHR mRNA and GH-binding, but an increase in plasma GH-binding sites (Finidori *et al.*, 1980; Maheshwari *et al.*, 1992b; Tonshoff *et al.*, 1994). This dichotomy may result from decreased renal clearance of the GHBP (due to decreased renal mass) rather than differential regulation of the GHBP and GHR. Conversely, both hepatic GHRs and serum GHBPs are reduced in cirrhotic patients and hepatectomized rats (Baumann *et al.*, 1989a; Amit *et al.*, 1990; Baruch *et al.*, 1991; Hattori *et al.*, 1992).

Patients with insulin-dependent diabetes mellitus are similarly GH-resistant, as IGF-I levels are reduced despite elevated concentrations of plasma GH. A deficiency in hepatic GH binding has been implicated, as hepatic GHRs are reduced in animal models of diabetes and GHBPs are low in insulin-dependent diabetics (Bornfeldt *et al.*, 1989; Mercado *et al.*, 1992; Maheshwari *et al.*, 1993). These changes are thought to be a direct result of insulinopenia, as insulin can restore GHR mRNA levels in diabetic rats and enhance hGH binding to adipocytes in hypophysectomized rats (Bryson & Baxter, 1986; Bornfeldt *et al.*, 1989; Massa *et al.*, 1993). However, other investigators failed to observe reduced hepatic GHR mRNA and GH-binding in diabetic rats (Bornfeldt *et al.*, 1989), thus post-receptor defects may also be causal in this form of GH resistance.

C. Inherited GH Resistance: Laron Type Dwarfism

The etiology of dwarfism is often linked to deficient or mutated growth hormone; however, dwarfism can also be associated with supranormal GH levels and GH resistance as in Laron type dwarfism (LTD) (Hull *et al.* 1993). LTD is an autosomal-recessive disorder characterised by severe growth retardation, IGF-I deficiency and other phenotypic symptoms of GH deficiency but normal or elevated serum concentrations of biologically active GH (Keret *et al.*, 1988; Russell *et al.*, 1989; Laron, 1993). These patients respond well to IGF-I (Laron *et al.*, 1991; Laron *et al.*, 1992; Kanety *et al.*, 1993) but are

unresponsive to exogenous GH (Spandoni *et al.*, 1988; Laron *et al.*, 1991), signifying GH resistance; thus, GH receptors may be dysfunctional in this disorder. Indeed, linkage analysis has demonstrated that LTD and GHR gene polymorphisms were co-inherited in 2 Mediterranean families in which the parents were consanguinous and had some children with LTD (Amselem *et al.*, 1989). The children with LTD were homozygous for the same DNA markers for which the parents were heterozygous.

A large number of LTD patients lack hepatic and serum GH binding activity (Eshet *et al.*, 1984; Aguirre *et al.*, 1990), suggesting a defect in the extracellular domain of the GHR. Indeed a variety of mutations have now been identified in the coding region of the extracellular domain by Southern Analysis and gene sequencing. These defects include deletions of exons 3, 5, and 6 (Godowski *et al.*, 1989), smaller, 2-residue deletions (Berg *et al.*, 1992), and nonsense mutations (Amselem *et al.*, 1991). Point mutations that create or destroy splice sites (Amselem *et al.*, 1992; Amselem *et al.*, 1993) or that alter residues important in GH binding (Amselem *et al.*, 1993) or possibly GHR trafficking (Duquesnoy *et al.*, 1991) can also result in the LTD phenotype. These mutations are summarized in Figure 1.4.

The molecular defects in the extracellular domain described above result in complete GH insensitivity. However, populations with low or normal levels of functional serum GHBPs and patients exhibiting only partial GH insensitivity have also been identified (Maheshwari *et al.*, 1992a). Defects in the regulatory regions of the GHR gene may account for some cases of low serum GHBP levels, as the pubertal rise in GHBP is absent in growth-impaired pygmies (Merimee, 1992). However, analysis of the pygmy GHR gene has only revealed an intronic defect of unknown signficance. Defects in the intracellular domain of the GHR could also result in decreased efficiency of signal transduction and varying degrees of growth impairment. Accordingly, 2 point mutations were recently identified in the GHR gene of a Laron dwarf (Kou *et al.*, 1993). However, the signficance of this finding is unknown, as both the patient and his mother were heterozygous for these mutations and the mother was not growth-deficient.

1.6 SIGNAL TRANSDUCTION

The geographic separation between membrane-bound receptors and cytoplasmic and nuclear sites of action necessitates the use of intracellular messenger systems. A number of second messengers have been implicated in GH action, including cAMP/protein kinase A, diacylglycerol/protein kinase C, calcium, and tyrosine kinases. Some of these pathways are summarized in Figure 1.7. Selective use of these heterogenous messenger systems may permit diverse effects of GH to be mediated by homologous receptors.

A. Tyrosine Kinases

Tyrosine kinase involvement in GH signal transduction is well-accepted, as GH stimulates tyrosine phosphorylation of the GHR and of cellular kinases (eg. ribosomal SP6 kinase, MAP kinase) and inhibitors of tyrosine phosphorylation block GH action (Stred *et al.*, 1992; Winston & Bertics, 1992; Campbell *et al.*, 1993; Campbell *et al.*, 1993). Although it was originally thought that the GHR itself may possess tyrosine kinase activity, it is now known that this enzyme activity resides in Janus kinase 2 (JAK2) (Argetsinger *et al.*, 1993). This kinase becomes closely associated with the GHR in response to GH binding and is instrumental in GH signal transduction (Argetsinger *et al.*, 1993). In particular, JAK2 is thought to mediate the transcriptional effects of GH on the c-fos, Spi2.1 and IGF-I genes.

GH may induce c-fos transcription activation via multiple pathways. First, GH binding induces formation of a GHR:JAK2 complex, tyrosine phosphorylation of JAK2 and the GHR, and activation of JAK2 kinase activity as described above (Argetsinger *et al.*, 1993). Activated JAK2 may then phosphorylate and activate the p91 component of the growth hormone-inducible DNA-binding factor (GHIF), which results in the phosphorylation, nuclear translocation and activation of the c-cis-inducible element (SIE). This transcription factor subsequently binds the c-fos promoter and stimulates c-fos production (Meyer *et al.*, 1994a; Meyer *et al.*, 1994b).

An alternate pathway mediating GH-induced c-fos production may also involve JAK2. In this cascade, GH activates MAP kinase activity, which, via ternary complex factors (VanderKuur *et al.*, 1994) and in synergy with the serum-response factor (SRF),

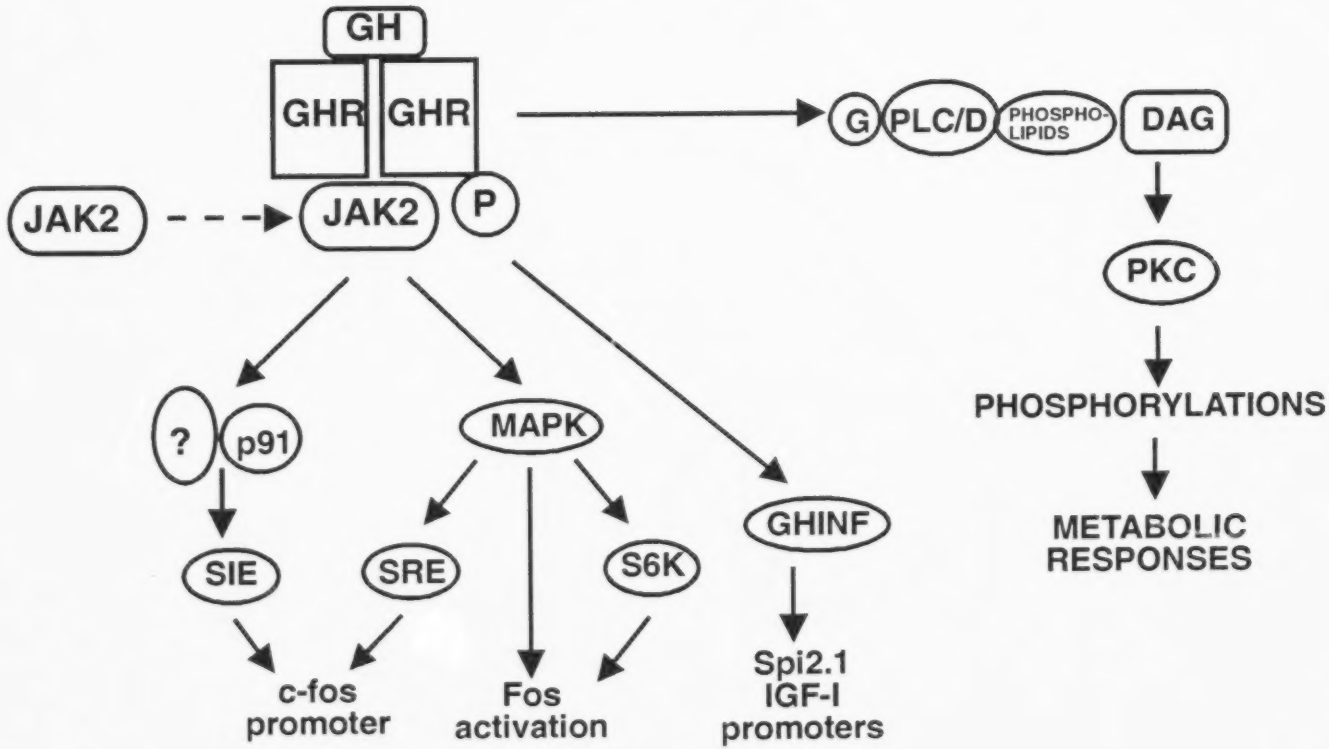

Figure 1.4. Putative signalling cascades involved in GH action. GH, growth hormone; GHR, growth hormone receptor; G, G-protein; PLC/D, phospholipase C/D; DAG, diacylglycerol; PKC, protein kinase C; JAK2, Janus kinase 2; MAPK, mitogen-activated protein kinase; S6K, SP6 ribosomal kinase; GHINF, GH-inducible nuclear factor; SIE, c-sis inducible element; SRE, serum-response element. Based on Roupas and Herington, 1994.

activates a serum response element (SRE). The delayed activation of MAPK as compared to JAK2 suggests that JAK2 may mediate GH-induced MAPK activation (Winston & Bertics, 1992; Argetsinger *et al.*, 1993). The SRE and the SIE described above then act at distinct sites in the c-fos promoter to stimulate c-fos gene transcription. GH may also post-translationally modify c-fos gene products, as GH activation of MAPK subsequently activates ribosomal SP6 kinase, and SP6 and MAPK phosphorylate and activate the Fos protein (Anderson, 1992; Anderson, 1993).

However, GHIF is not involved in GH-induced activation of the Spi2.1 and possibly IGF-I genes. Instead, GH effects are mediated by a GH-initiating nuclear factor (GHINF), which binds the GH response element of the Spi2.1 gene and perhaps a homologous motif in the IGF-I gene (Berry *et al.*, 1994; Enberg *et al.*, 1994; Thomas & Rotwein, 1994).

B. Calcium

There is evidence for a role of calcium in some but not all the actions of GH. For example, modulations in intracellular calcium are necessary for GH-induced insulin synthesis (Billestrup & Bouchelouche, 1994) and lipolysis (Campbell & Scanes, 1988) but are not required for the insulin-like effects of GH or GH-mediated c-fos synthesis. Although the mechanism by which GH induces calcium fluxes is not known, it is thought to involve RNA and protein synthesis (Schwartz *et al.*, 1992).

C. Proto-Oncogenes

Proto-oncogenes such as c-fos are common mediators of somatogenic and proliferative effects of growth factors. As described, GH is also known to stimulate c-fos expression, phosphorylation and activation in a variety of cell lines (Doglio *et al.*, 1989; Gurland *et al.*, 1990; Barcellini-Couget *et al.*, 1993) through tyrosine kinase-dependent and protein kinase C-dependent pathways (Doglio *et al.*, 1989; Gurland *et al.*, 1990). However, GH induces expression of c-fos in differentiating and non-differentiating cells (Sumantran *et al.*, 1992); thus, c-fos induction alone is not sufficient to activate the full biological response.

D. Protein Kinase C

Protein kinase C (PKC) may be an additional player in a subset of GH actions, as GH stimulates phospholipase C and phospholipase D activation, glycerophospholipid hydroloysis, diacylglycerol release and PKC activation (Doglio *et al.*, 1989; Rogers & Hammerman, 1989; Roupas & Herington, 1994). PKC inhibitors prevent GH-induced activation of S6 kinases (Anderson, 1992) and GH-induced synthesis of lipoprotein lipase (Pradines-Figueres *et al.*, 1990), suggesting PKC involvement in these processes. The role of PKC in c-fos synthesis and MAPK activation remains controversial (Doglio *et al.*, 1989; Anderson, 1992; Barcellini-Couget *et al.*, 1993). PKC may also mediate some insulin-like and diabetogenic effects of GH on lipid metabolism (Smal *et al.*, 1989; Goodman *et al.*, 1990; Gorin *et al.*, 1992).

E. Protein Kinase A

GH may exert minor effects on the cAMP/PKA signalling pathway, as GH reduces the accumulation of cAMP in the presence of epinephrine in hypophysectomized rat adipose tissue (Birnbaum & Goodman, 1976). This interaction may be involved in the antilipolytic effect of GH, as GH partially suppresses lipolysis induced by epinephrine and glucagon (Goodman, 1970; Campbell & Scanes, 1987) which act via cAMP. Moreover, cAMP may mediate GH-induced upregulation of PEPCK gene transcription (Donkin & Etherton, 1994).

F. Direct Genomic Effects

The close association between GHR/GHBP immunoreactivity and heterochromatin in rat hepatic cells suggests that the GHR/GHBP may directly mediate some nuclear effects of GH (reviewed by Harvey & Hull, 1994a). However, the GHR/GHBP does not contain consensus sequences for DNA-binding proteins, thus nuclear PKC and tyrosine kinases are likely mediators of the nuclear effects of GH. Alternatively, GHR/GHBPs may act as intracellular shuttles, enabling transport of GH from the plasma membrane to the nuclear compartment. This hypothesis is supported by the rapid nuclear translocation of long and short forms of the GHR in response to GH binding (Goodman *et al.*, 1994;

Lobie *et al.*, 1994). Moreover, GH is transported to the nucleus as an intact molecule (Lobie *et al.*, 1994) and thus could retain its bioactivity. GHBP:GH complexes may actually enter the nuclear compartment, as the hydrophilic tail of the GHBP contains 4 amino acids that may interact with the nuclear pore complex.

1.8. HYPOTHESIS AND AIMS

A. Hypothesis

It is now well-established that GH resistance in many clinical conditions results from a deficiency or defect in tissue GH receptors. Consequently, the inability of some tissues to respond to GH stimulation could indicate dysfunctional or absent growth hormone receptors. GH resistance and short stature in animals may similarly result from GH receptor anomalies. These two hypotheses were therefore separately addressed in a series of studies described below.

B. Specific Aims

I. GH-resistant Tissues

a. Pituitary Growth Hormone Receptors?

Although the pituitary gland is exposed to very high concentrations of growth hormone, the pituitary has historically been considered to be resistant to GH action. The possibility that the GH receptor gene may not be expressed in the adenohypophysis was therefore examined in domestic fowl, in which GH-secreting cells are geographically separated from other pituitary cell types.

b. GH-binding Proteins in Pituitary Cells: Synthesis and Secretion in Tumorous Somatolactotrophs

The growth hormone (GH) receptor (GHR) gene is expressed in somatotroph and lactotroph cells of the rat adenohypophysis, in which GHR/GH-binding protein (GHBP) immunoreactivity is primarily located in the secretory granules. The possibility that the GHR gene may be similarly expressed in GH_3 cells was therefore investigated, since these tumorous rat pituitary cells are used as models of GH- and prolactin-secreting cells but

lack secretory granules.

c. Thyroid Gland: A GH Target Site?

Although it is well-established that GH affects thyroid function, hitherto this has been solely to be due to effects on the peripheral metabolism of thyroid hormones. Indeed, while lactogenic GH transgenes stimulate thryoidal function, somatogenic transgenes are ineffective. The possibility that the thyroid gland may lack GH receptors was therefore assessed.

ii. GH-resistant Animals

a. Sex-linked Dwarf Chicken

Growth hormone (GH) resistance in Laron dwarfism is thought to be due to a defect in the GH-receptor gene; both point mutations and deletions have been identified. The possibility that a defect in GH-receptor gene expression may also account for the sex-linked dwarfism in domestic fowl was therefore investigated, since its etiology is unknown.

b. Guinea Pigs

The presence of growth hormone receptor (GHR) gene transcripts and growth hormone (GH) binding sites in guinea pigs suggests normal expression and translation of the GHR gene in these animals. Guinea pigs are, however, resistant to GH action and apparently lack the circulating GH-binding proteins (GHBPs) that may result from alternate splicing of the GHR message or from cleavage of the extracellular binding domain of membrane GHR's. The paradoxical absence of circulating GHBP's was therefore re-evaluated.

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CHAPTER 2: EXPRESSION OF THE GROWTH HORMONE RECEPTOR GENE IN CHICKEN PITUITARY GLANDS[1]

2.1 INTRODUCTION

The autoregulation of growth hormone (GH) secretion is thought to be primarily regulated at hypothalamic sites, by increased somatostatin (SRIF) release and/or by decreased GH-releasing factor (GRF) secretion (Rogers, Vician, Steiner & Clifton, 1988; Cella, De Gennaro Collona, Locatelli *et al.* 1990; Müller, 1990). The demonstration of GH receptors in the brain (Stern, Miller, Resnick & Morgane, 1976; Van Houten, Posner & Walsh, 1980; Mathews, Enberg & Norstedt, 1989; Fraser, Attardo & Harvey, 1990; Waters, Barnard, Lobie *et al.*, 1990), particularly in GRF- and SRIF-containing nuclei (Burton, Kabigting, Clifton & Steiner, 1991), supports this possibility. Extra-pituitary sites of GH autoregulation are also suggested by the inability of exogenous GH to directly inhibit GH release from pig (Glenn, 1986), sheep (de Zegher, Bettendorf, Grumbach, & Kaplan 1990), rat (Richman, Weiss, Hochberg & Florini, 1981; Lamberts & Oosterom, 1985; Kraicer, Lussier, Moor & Cowan, 1988), and chicken (Harvey & Baidwan, 1990) pituitary glands or to bind to rat pituitary membranes (Goodyer, De Stephano, Lai *et al.*, 1984; Fraser, Siminoski & Harvey, 1991). It is therefore of interest that GH receptor mRNA is present in rat, rabbit, and human pituitary glands (Fraser *et al.*, 1991) and that GH may stimulate the production of insulin-like growth factors (IGF's) in the rat pituitary (Fagin, Brown & Melmed, 1988). Moreover, whereas exogenous GH has no direct effect on GRF-induced GH release from bovine pituitary glands, it directly inhibits basal GH release (Glenn, 1986; Rosenthal, Silverman & Wehrenberg, 1991). The blunting of the endogenous GH response to GRF by exogenous GH in human subjects in which lipolysis and SRIF secretion are pharmacologically blocked (Pointiroli, Lanzi, Monti *et al.*, 1991) also suggests autoinhibition of GH secretion at the pituitary level. The possibility that GH

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may have pituitary sites of action was therefore investigated in the present study, by determination of GH receptor (GHR) gene expression and GH-binding to chicken adenohypophyseal tissue.

2.2. MATERIALS AND METHODS

A. Northern Blot Analysis

GH receptor gene expression in the chicken (White Leghorn) pituitary gland was established by Northern Blot analysis and by the polymerase chain reaction (PCR). Total cellular RNA was extracted from the caudal and/or cephalic lobes of the chicken adenohypophysis (Mikami & Takahashi, 1987) in 5.5 mol/l guanidinium thiocyanate, containing 25 mmol/l sodium citrate and 0.5% (w/v) sodium lauryl sarcosine (pH 7) and collected after centrifugation through a bed of caesium trifluoroacetic acid (Pharmacia Fine Chemicals, Uppsala, Sweden, density 1.51) containing 0.1 mol/l EDTA, pH 7.0 (Fraser *et al.* 1991). The RNA was quantified spectrophotometrically and 5 µg (liver) or 20 µg (pituitary) aliquots were subjected to electrophoresis in 1% (w/v) agarose and 3.1% (v/v) formaldehyde gels and transferred to Hybond N nylon membranes (Amersham, Mississauga, Ontario, Canada) by capillarity. A rabbit GHR (rGHR) complementary RNA (cRNA) probe was constructed from a 638 bp *Bam*HI/*Eco*RI fragment of the rabbit GHR cDNA sequence (Leung, Spencer, Cachianes *et al.* 1987). The plasmid pRGEM32 (Kawasaki, 1990) containing this sequence was linearized by *Bam*HI digestion (BRL, Burlington, Ontario, Canada) and transcribed by T7 - polymerase (BRL) in the presence of [^{32}P] αCTP (800 uCi/mmol; New England Nuclear, Mississauga, Ontario, Canada). The probe was hybridized with the immobilized RNA in 50% formamide (containing 0.75 mol/l NaCl, 25 mmol/l PIPES and 25 mmol/l EDTA, 0.1% (w/v) Ficoll, 0.1% (w/v) BSA, 0.2% (w/v) sodium dodecyl sulphate (SDS), 0.1% (w/v) polyvinylpyrrolidine, 10 ug/ml salmon sperm DNA and 10 ug/ml yeast tRNA, pH 6.8) for 12 h at 68°C, following a 3 h. incubation in the absence of the probe. The nylon membranes were then washed twice in each of 2x, 0.5x and 0.05x SSC (1xSSC = 0.015 mol/l sodium citrate and 0.15 mol/l NaCl, pH 7.2) (containing 0.2% (w/v) SDS) at room temperature, 68°C and 68°C, respectively, prior to exposure to Kodak X-OMAT-AR film (Kodak, Rochester, NY,

USA) for 3 days. As a negative control, a sense probe was prepared by transcription of the linearized *Bam*HI/*Eco*RI rGHR fragment in the presence of SP6 - polymerase (BRL) and hybridized with the blot as described above.

B. Polymerase Chain Reaction

Total RNA extracted from the chicken pituitary and rabbit liver was reverse transcribed by Superscript (100 U, BRL) in the presence of 100 pmoles of oligodeoxythymine primer (Boehringer Mannheim, Dorval, Quebec, Canada), excess nucleotides (10 mmol/l each of dCTP, dTTP, dATP and dGTP, Boehringer Mannheim) and 5x H-RT buffer (BRL). Reactions were also prepared in the absence of RNA or Superscript to serve as negative controls. The reactions were diluted with double-distilled water (50:1 v/v) and an aliquot of each (0.5% of total volume) was added to a PCR (Kawasaki, 1990) cocktail containing 5' - oligomer sense (CCTCGATTTGGATACCATATTGTGTTAAGC) and/or 3' -oligomer antisense (CTGTTACGGCCAGCCCACACACTCCGAAG) primers (Nucleotide Synthesis Lab, University of Alberta, Edmonton, Alberta, Canada) for the extracellular and transmembrane domains of chicken liver GHR cDNA (15 pmol of each) (Figure 2.1), deoxynucleotides (200 umol/l of each), 1x PCR reaction buffer (50 mmol/l KCl, 10 mmol/l Tris-C1, pH 8.4, 1.5 mmol/l $MgC1_2$, and 20 ug/ml gelatin) and *Thermus aquaticus (Taq)* DNA polymerase (5 U, BRL). The primers were based on the known sequence of the extracellular domain of the chicken liver GHR (Burnside, Liou & Cogburn, 1991). The mixture was overlayed with mineral oil (v/v), and heat denatured at 94°C for 2 min. before 30 cycles of annealing (at 50°C for 1 min), extension (at 72°C for 3 min) and denaturing (at 94°C for 1 min) in a genetic thermal cycler (Precision Instruments, Chicago, IL). The PCR product was further characterized by digestion with the restriction enzymes *Nco*I, ***Bst***NI and *Bam*HI (5 units/ug DNA, BRL) for 2 h at 37°C. The reaction products were electrophoresed in 1.4% agarose ethidium bromide-stained minigels and compared with *Hae*III-digested pUC 18 size markers or *Eco*RI/*Bam*HI-digested λ size markers. RNA extracted from the caudal and cephalic lobes of the chicken pituitary was similarly reverse transcribed and amplified by PCR in the presence of 5'-oligomer sense

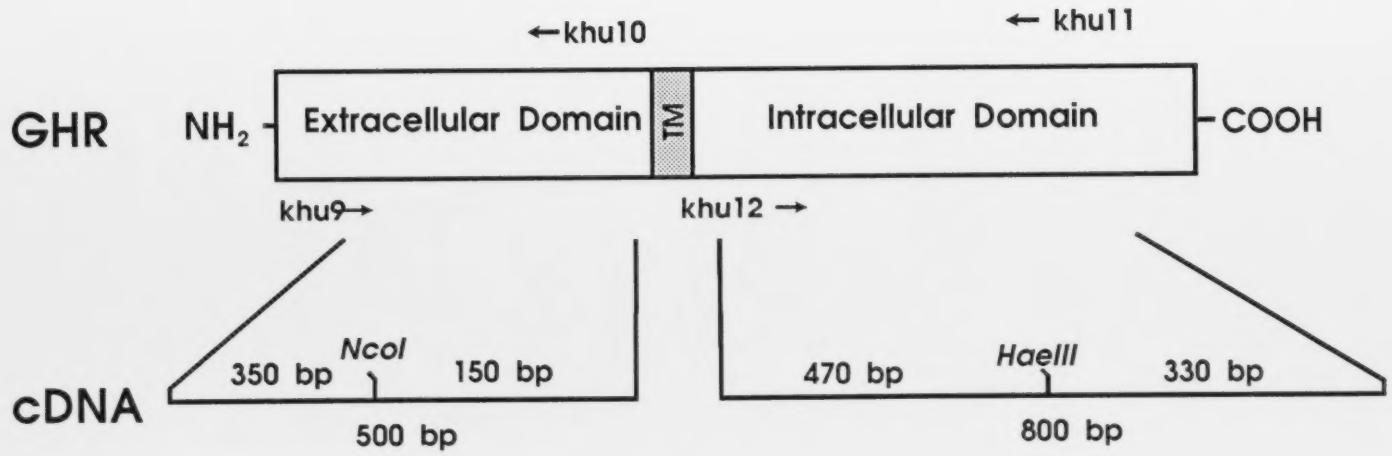

Figure 2.1. Sites of primer annealing and restriction enzyme digestion on the GHR cDNA (TM = transmembrane).

(CTGCGGCCGCAGGACCAGTTCCAAAGATTAA) and 3'-oligomer antisense primers (AAGCGGCCGCGGCAGTAGTGGTAAGGCTTTC) for the intracellular sequences of the chicken liver GHR cDNA (Burnside *et al.* 1991) (Figure 2.1). Restriction enzyme digestion was performed with *Eco*RI, *Bst*NI and *Hae*III (5 units/ug DNA, BRL) for 2 h at 37°C.

C. Binding Studies

The possible translation of the GHR message was qualitatively assessed by determination of GH-binding sites in the chicken pituitary gland. The glands were dissected from the heads of freshly killed broiler fowl, obtained from a local slaughterhouse (Lillydale, Edmonton, Alberta, Canada) and homogenized in ice-cold 0.2 mol/l sucrose. The homogenate was then centrifuged (10 000 x g for 90 min at 4°C) and the plasma membranes were precipitated from the cytosolic fraction by ultracentrifugation (100 000 x g for 90 min at 4°C). Nuclear membranes were extracted from a 0.32 mol/l sucrose (containing 3 mmol/l $MgCl_2$) homegenate of the pituitary glands after centrifugation (700g for 10 min, at 4°C). The pellet was resuspended in 1.0 ml of the same buffer (buffer A) and in 2.0 ml 2.1 mol/l sucrose (containing 2 mmol/l $MgCl_2$, buffer B) and layered onto 2 ml buffer B prior to ultracentrifugation (50 000 g for 60 min. at 4°C) (Widnell & Tata, 1964). The cellular fractions had no GH immunoreactivity (<1 pg/mg protein) in an homologous GH radioimmunoassay (Harvey & Scanes, 1977), and were diluted with ice-cold 25 mmol/l Tris-HC1 buffer (containing 0.2% (w/v) bacitracin, 0.1% (w/v) bovine serum albumin and 10 mmol/l $CaCl_2$, pH 7.5). Aliquots of the cellular fractions (derived from 100-200 mg wet weight pituitary tissue) were incubated for 5 h at room temperature with approximately 100 000 cpm ^{125}I-labelled (specific activity 40-50 uCi/ug) ovine GH (oGH-14, biological grade, 1.4 IU/mg, NIADKD, Bethesda, MD, USA), in the presence or absence of unlabelled oGH (10 ug/ml), to determine non-specific binding (Fraser *et al.* 1990). The tracer was >95% precipitable with excess 10% (w/v) trichloroacetic acid and eluted as a single monomeric peak when chromatographed (Harvey & Scanes, 1977) on Sephadex G-100 (Pharmacia Fine Chemicals). The tracer was used within 4 - 5 days of iodonation with fresh $Na^{125}I$

(Edmonton Radiopharmaceutical Centre, Edmonton, Canada). The separation of free and bound GH was achieved at 4°C by gel filtration of the incubation media on 1x50 cm columns of Sephadex G-100 in a 10 mmol/l sodium phosphate elution buffer (containing 10 mmol/l EDTA, 150 mmol/l NaCl, 0.1% BSA (w/v), 0.1% (w/v) sodium azide and 20% (v/v) glycerol, pH 7.6), at a flow rate of 1.0 ml/min (Snow, Shaw, Winer & Baumann, 1990). Fractions (0.5 ml) were collected and aliquots (100 µl) were counted for radioactivity. The radioactive peaks were characterized by comparison with ^{125}I-labelled oGH and Na^{125}I and with the elution of molecular mass markers (blue dextran, MW = 2000 kDa, aldolase, MW = 150 kDa, collagenase, MW - 105 kDa, BSA, MW - 66 kDa, carbonic anhydrase, MW = 29 kDa, and cytochrome c, MW = 12 kDa, Sigma Chemical Company, St. Louis, MO, USA) that were monitored spectophotometrically at 280 nm. For comparative purposes, 500 µl chicken plasma was fractionated and incubated with the tracer to determine GH-binding to circulating proteins (Snow *et al.* 1990). As the liver is an established site of GH action (Waters *et al.*. 1990), the binding of GH to chicken liver membranes was also compared with GH-binding to pituitary tissues. At least five Sephadex columns were used to separate the radioactivity resulting from the incubation of radiolabelled GH with each tissue fraction and significant differences in the size of the radioactive peaks were determined by Student's t-test.

2.3. RESULTS

A. Molecular Studies

Hybridization with the cRNA probe occurred with total RNA extracted from the pituitary glands of adult (>24 weeks of age) chickens (Figure 2.2). The hybridization was with a single RNA moiety of approximately 4.4 kb and of similar size to that in RNA extracted from chicken liver. This hybridization was specific since no signal was detected using the sense probe (data not shown).

In the presence of 3′- and 5′-oligonucleotide primers for the extracellular and transmembrane domains of the chicken liver GHR cDNA (Figure 2.1), cDNA synthesized from chicken pituitary extracts was amplified by PCR and stained with ethidium bromide. A single band approximately 500 bp in size comigrated with the 499 bp PCR product

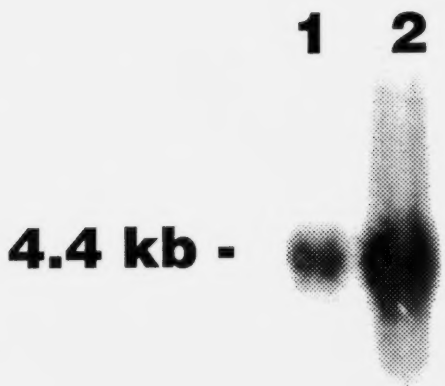

Figure 2.2. Hybridization of a complementary RNA probe for the rabbit GH receptor cDNA with total cellular RNA extracted from liver (lane 1) and pituitary glands (lane 2) of adult (> 24 weeks of age) chickens.

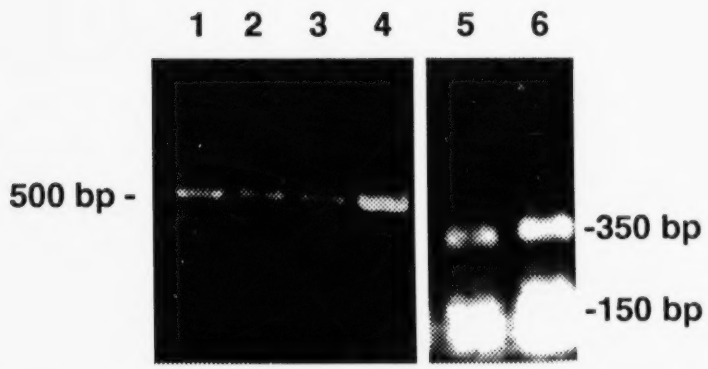

Figure 2.3. Ethidium bromide-stained 1.4% agarose gel through which cDNA was electrophoresed. The cDNA was reverse transcribed from total RNA extracted from 1-week (lane 1), 16-week (lane 2) and adult (>24 weeks of age)(lane 3) anterior pituitary glands and adult liver (lane 4) of Cornell White Leghorns and amplified by PCR in the presence of oligonucleotide primers khu 9 and khu 10. NcoI digests of amplified adult pituitary (lane 5) and adult liver (lane 6) are also shown. The sizes of the fragments were determined by comparison with the migration of Hae-III-digested pUC18 and EcoRI/BamHI-digested lambda size markers.

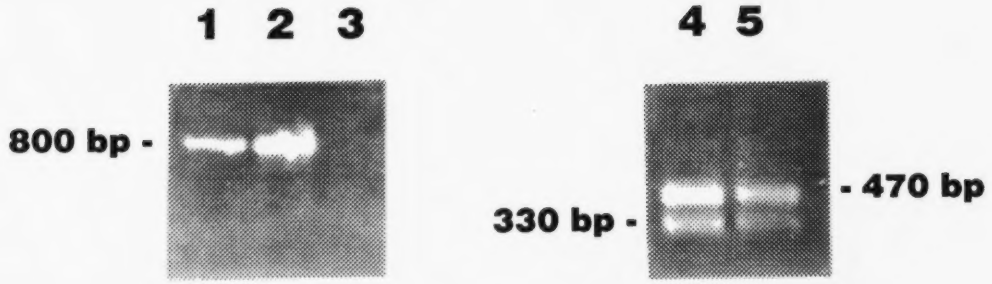

Figure 2.4. Ethidium bromide-stained 1.4% agarose gel through which cDNA was electrophoresed. The cDNA was reverse transcribed from total RNA extracted from the cephalic (lane 1) and caudal (lane 2) lobes of chicken pituitary glands of Cornell White Leghorns and amplified by PCR in the presence of oligonucleotide primers khu 11 and 12. Lane 3 contains a negative control in which no RNA was added to the reverse transcription mixture, and the HaeIII digests of the amplified cephalic and caudal lobes are shown in lanes 4 and 5, respectively. The sizes of the fragments were determined by comparison with the positions of HaeIII-digested pUC18 and EcoRI/BamHI-digested lambda size markers.

derived from chicken liver cDNA (Figure 2.3). When chicken pituitary cDNA was digested with *Nco*I, two bands of approximately 350 bp and 150 bp were observed, as would be expected for the digestion of the cGHR cDNA fragment. Digestion of the cDNA with *Bam*HI and *Bst*NI also produced fragments of the expected size (325 bp and 171 bp, 271 bp and 224 bp, respectively) (data not shown). Reverse transcription of RNA extracted from caudal and cephalic lobes of the chicken pituitary gland and amplification of the resulting cDNA with chicken GHR 3′- and 5′-oligonucleotide primers revealed a single electrophoretic band of approximately 800 bp (Figure 2.4). As expected, *Eco*RI digestion of the cephalic and caudal lobe PCR products produced fragments of approximately 530 bp and 275 bp. Moieties of predicted size were also observed following digestion with *Hae*III (469 bp and 337 bp) and *Bst*NI (200 bp and 606 bp) (data not shown). In both PCR reactions, these fragments were not generated when either of the primers was omitted from the reaction or when the RNA was not transcribed by Superscript or was absent.

B. Binding Studies

After the incubation of chicken liver membranes with ^{125}I-labelled GH, three peaks of radioactivity were separated on Sephadex G-100 (Figure 2.5a). The second peak (54.2% of radioactivity) co-eluted with the tracer and the third peak (19.9% of radioactivity) co-eluted with Na^{125}I, and (despite the presence of bacitracin) presumably reflected tracer degradation during the 5 h incubation. The first peak had an approximate molecular mass of 80-86 kDa and accounted for 25.9% of the total radioactivity separated on the Sephadex columns. The incubation of liver membranes with unlabelled GH prior to gel filtration reduced ($p<0.01$) the radioactivity in the first peak by 52% but had no significant effect on the amount of radioactivity eluted in the other peaks. Similar peaks of radioactivity were also observed after the incubation of ^{125}I-labelled oGH with pituitary plasma membranes (Figure 2.5b) and nuclear membranes (Figure 2.5c). In both cases the amount of radioactivity eluting in the first peak was significantly reduced ($p<0.01$ by 51% for plasma membranes and by 68.7% for nuclear membranes) after the tissue fractions were incubated in the presence of excess unlabelled GH Radiolabelled oGH was also

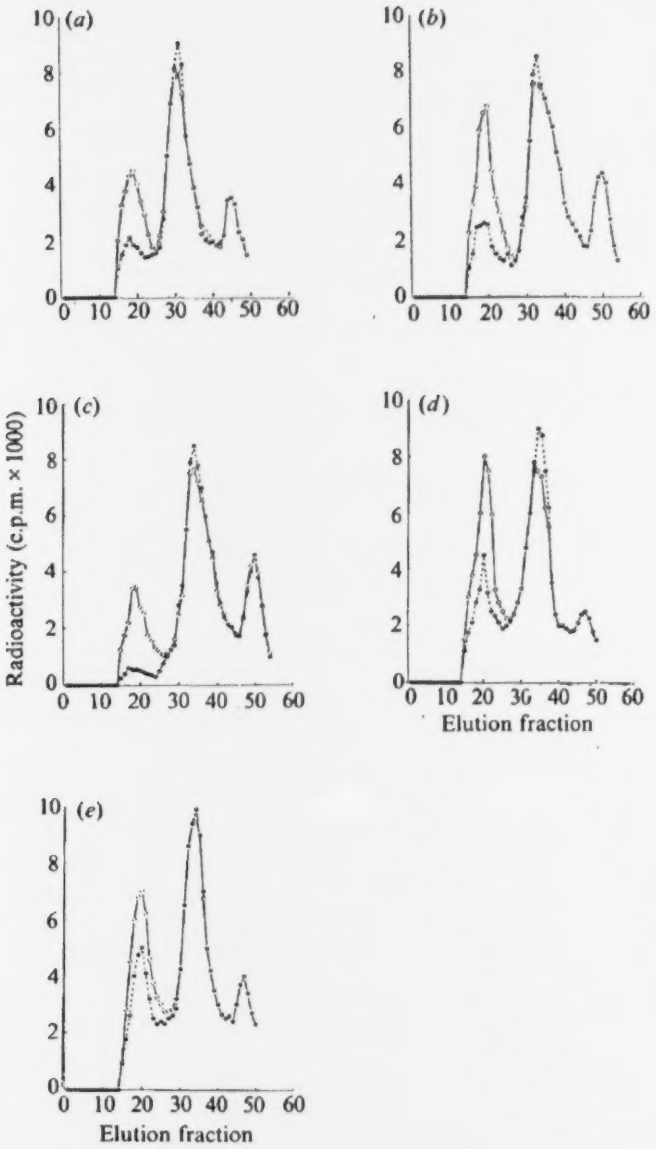

Figure 2.5. Typical elution profile of ^{125}I-labelled ovine GH from a 1 x 50 cm column of Sephadex G-100 after a 5 h incubation of the tracer in the absence (open symbols) or presence (solid symbols) of unlabelled GH with chicken (a) liver plasma membranes, (b) pituitary plasma membranes, (c) pituitary nuclear membranes, (d) pituitary cytosol and (e) plasma.

bound to pituitary cytosol (Figure 2.5d) and chicken plasma (Figure 2.5e), although to moieties of lower molecular size (radioactive peaks of approximately 72 kDa). In the presence of excess unlabelled GH the radioactivity in the first peak was reduced by 34% for the cytosolic fraction and by 43.1% for plasma.

2.4. DISCUSSION

These results indicate the expression and translation of the GHR gene in chicken pituitary glands. While GH receptor mRNA of comparable size has recently been identified in mammalian pituitary glands (Fraser *et al.*, 1991) this is the first demonstration of GH-binding sites in adenohypophyseal tissue, although it is well established that the pituitary accumulates labelled GH after systemic administration (Stern *et al.*, 1976). Since GH stimulates the production of insulin-like growth factor - I (Fagin *et al.*, 1988) and 5' monodeiodonase activity (Geelhoed-Duijvestijn, Roelfsema, Schröder-van der Elst *et al.*, 1992) in the rat pituitary gland, these binding sites may indicate the presence of GH receptors in the chicken adenohypophysis. The possibility that the pituitary gland may be a target site for GH action is also supported by the recent demonstration that an antiserum to GH markedly reduced pituitary weight and impaired pituitary-thyroid, pituitary-adrenal and pituitary-gonadal activity in neonatal rats (Flint, Gardner, Akinsanya & Wynick 1992). Autoregulatory actions of GH on the release of bovine pituitary GH have also been reported (Glenn 1986; Rosenthal *et al.*, 1991).

These pituitary GH-binding sites were qualitatively detected by chromatographic separation of bound radioactivity, but accounted for only < 20 % of total tracer binding. This minimal level of GH binding may partly account for the inability of Posner, Kelly, Shiu & Friesen (1974), Goodyer *et al.* (1984), and Fraser *et al.* (1991) to previously demonstrate GH-binding to rat pituitary membranes. This may, however, also reflect the different methodologies used for the separation of bound hormone (centrifugation vs. gel filtration), as GH-binding sites in the chicken pituitary were not evident when the bound and free hormone were separated by centrifugation (S. Harvey, unpublished observations). The greater discrimination of chromatographic separation procedures was also shown by Barnard, Bundesen, Rylatt & Waters (1984). These authors found that polyethylene

glycol precipitation and centrifugation underestimated the amount of large molecular weight (≥ 100 kDa) moieties present in [^{125}I] GH-GHR incubations, since these moities were detected after gel filtration of the supernatant fractions.

The presence of GH-binding sites may also have been missed in previous mammalian studies because of saturable binding by undissociated pituitary GH The expression of the GH receptor gene in the rat brain is reported to decline with postnatal age in a manner opposite to that seen in the liver and muscle (Waters *et al.,* 1990), whereas the content of pituitary GH increases during ontogeny (Rieutort, 1976; Walker, Dissault, Alvarado-Urbina & Dupont, 1977). In contrast, the GH content of the pituitary declines in old birds (Hoshino, Suzuki & Yamamoto, 1980; Foltzer, Harvey & Mialhe, 1987), in which unoccupied GH receptors are therefore more likely to be present.

In addition to the plasma membranes, GH-binding sites in the pituitary gland were also detected (but not quantified) in the cytosol and in nuclear membranes. The moiety in the plasma membrane that bound labelled GH had an approximate molecular mass of 60 kDa. This is smaller than the calculated molecular mass (70 kDa) and the actual molecular size (approximately 130 kDa) of the membrane-bound rabbit liver GH receptor, which is glycosylated and bound to ubiquitin (Kelly, Djiane, Postel-Vinay & Edery, 1990). This size is, however, similar to that reported for major GH-binding subunits in rat, rabbit and human liver membranes (Hughes, Simpson & Friesen, 1983; Ymer & Herington, 1984; Hocquette, Postel-Vinay, Djiane *et al.,* 1990). The size of these chicken GH-binding proteins may indicate extensive proteolysis during the 5 h incubation or that they are not glycosylated or linked to ubiquitin. These membrane-bound binding sites were, however, of larger molecular size than those in pituitary cytosol, which were comparable with the 50 kDa binding proteins found in mammalian liver and kidney tissue (Barnard & Waters, 1986; Leung *et al.,* 1987; Waters *et al.,* 1990). The binding of labelled GH to these fractions was only displaced 50-70% by excess unlabelled GH This residual radioactivity may represent extensive non-specific binding, but could be due to tracer binding to a low affinity binding protein that is far from saturated at the dose of GH used to saturate high affinity binding proteins (Baumann, 1991). It is however, also possible that the residual radioactivity is partly due to aggregated tracer (Baumann, 1991).

The widespread distribution of GH-binding sites within pituitary cells is of interest, although immunoreactive GH receptors have also recently been located in the cytosol and nuclei of gastrointestinal and gonadal cells (Lobie, Breipohl, Garcia-Aragon & Waters, 1990; Lobie, Breipohl & Waters, 1990). Although primarily located in the microsomal fractions, nuclear fractions have also been found to express GH receptors in the human liver (Hocquette, Postel-Vinay, Kayser *et al.*, 1989). It is therefore possible that GH may have intracrine roles (Logan, 1990) within the pituitary gland. Moreover, as GH receptor mRNA was demonstrated in the cephalic lobe of the chicken pituitary gland, in which somatotrophs are lacking, and in the caudal lobe, in which somatotrophs are the most abundant cell type (Mikami & Takahashi 1987), GH may have autocrine actions and paracrine effects on somatotroph and non-somatotroph cells. Paracrine interactions between pituitary cells are well established and are important in the regulation of GH secretion (Denef, Baes & Schramme, 1986; Carmeliet & Denef, 1989). It is therefore possible that GH may act on neighbouring cells in an ultrashort-loop feedback manner. The existence of GH receptors in non-somatotroph cells has also been suggested by the presence of GH receptor mRNA in the posterior and intermediate lobes of the rat and rabbit pituitary glands (Fraser *et al.*, 1991).

The demonstration of GH receptor mRNA in the caudal lobe of the chicken pituitary suggests that GH-secreting cells may also synthesize GH-binding proteins. Pituitary GH has been isolated in aggregated forms, which have been thought to reflect protein-bound monomeric GH (Herington, Stevenson & Ymer, 1988). The pituitary gland has therefore been suggested (Ymer & Herington, 1985) to be a site for the synthesis of the GH-binding protein found in the rat, rabbit and human plasma (Baumann, 1990). The circulating GH-binding protein is almost identical to cytosolic GH receptors and the extracellular domain of the membrane bound GH receptor and is transcribed from the GH receptor gene (Barnard & Waters, 1986; Leung *et al.*, 1987; Baumann, 1990; Waters *et al.*, 1990). It is possible that the GH-binding sites in the chicken pituitary gland may include the binding protein identified in chicken plasma (Figure 2.5), as one set of oligonucleotide primers used in the PCR were complementary to the extracellular domain of the GH receptor cDNA. The existence of a plasma GH-binding protein in chickens

has also been reported by Vasilatos-Younken, Andersen, Rosebrough *et al.*, (1991), on the basis of interference in a chicken GH radioimmunoassay. If the GH-binding protein is expressed in the pituitary gland this could indicate pleurifunctional roles of GH receptor gene expression, since GH-binding to a protein rather than to a receptor could inhibit GH action or protect against GH degradation (Baumann, Amburn & Buchanan, 1987; Lim, Spencer, McKay & Waters, 1990). It is, however, uncertain if expression of the GH receptor gene in the adenohypophysis is for either or both the GH receptor and plasma binding protein, as the cRNA probe used hybridizes with RNA sequences that code for the extracellular, transmembrane and intracellular domains of the GH receptor.

In summary, these results demonstrate binding proteins within the pituitary for an adenohypophyseal hormone. This is a novel finding for GH, although it is not without precedent, since Kogawa, Nakamura, Sugino *et al.*, (1991) recently identified a binding protein in mammalian pituitaries for activin, which is secreted locally and has autocrine roles within the pituitary gland (Corrigan, Bilezikjian, Caroll *et al.*, 1991).

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CHAPTER 3: GROWTH HORMONE RECEPTOR/BINDING PROTEIN SYNTHESIS AND RELEASE IN TUMOROUS SOMATOLACTOTROPHS[1]

3.1 INTRODUCTION

The pituitary gland has traditionally been thought to be resistant to the effects of growth hormone (GH), as GH is unable to autoregulate its secretion from pituitary glands of rats, mice, pigs and chickens (Richman *et al.*, 1981; Lamberts & Oosterom, 1985; Glenn, 1986; Kraicer *et al.*, 1988; Harvey & Baidwan, 1990; de Zegher *et al.*, 1990). This GH-resistance was thought to result from a lack of tissue GH receptors (Goodyer *et al.*, 1984; Fraser *et al.*, 1991). The GH receptor (GHR) gene is, however, now known to be expressed in the pituitary gland, in which GH-binding sites have been detected in plasma and nuclear membrane and in cytosolic compartments (Fraser & Harvey, 1992; Hull *et al.*, 1992; Harvey *et al.*, 1993). These binding sites may, however, reflect the ubiquitous presence of GH-binding proteins (GHBPs) rather than authentic receptors. Although GHR immunoreactivity is abundantly present in all pituitary cell types (Harvey *et al.*, 1993), much of this immunoreactivity is due to the cross-reactivity of GHBP, which is identical to the extracellular domain of the GHR (Baumbach *et al.*, 1989). Moreover, as most of the GHR immunoreactivity is located within secretory granules of adenohypophyseal cells, it is still unclear if these cells are target sites for GH action.

In contrast with normal rat somatotrophs, GH release from tumorous somatolactotrophs (GH_3 cells), that secrete both GH and prolactin, is characterized by an autoregulatory mechanism (Lapp *et al.*, 1989; Stachura *et al.*, 1990). A GH moiety may thus act in intracrine, autocrine or paracrine ways to inhibit GH secretion, although this moiety is structurally distinct from 22 kDa rat GH (Lapp *et al.*, 1989; Stachura *et al.*, 1990). These tumorous cells, which are commonly used as models for the study of GH

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and prolactin secretion (Hinkle, 1984; Gourdji *et al.*, 1982), may thus differ from normal somatotrophs in GHR gene expression.

Cloned lines of pituitary tumor cells are uniquely suited to the study of receptors, because of their homogeneity and unlimited availability (Hinkle, 1984). They are, however, an imperfect model of GH and prolactin secretion, since their interactions with some regulatory factors differ from those of normal GH- and prolactin-releasing cells. Indeed, they may lack functional receptors for dopamine and GH-releasing factor and have an overabundance of thyrotropin-releasing hormone receptors (reviewed by Gourdji *et al.*, 1982; Harvey& Scanes, 1979). The feedback inhibition of GH secretion by these tumorous cells could thus be anomalous and reflect a difference in GHR gene expression. Alternate transcription of the GHR gene, which normally occurs in pituitary cells and leads to the production of GHBPs may, for instance, not occur in tumorous somatolactotrophs. Since GHBPs compete with GHRs for GH and inhibit GH actions *in vitro* (Lim *et al.*, 1990; Amit *et al.*, 1992; Mannor *et al.*, 1991), a GHBP deficiency could sensitize GH_3 cells to GH action. The possibility that GHR gene expression may be anomalous in GH_3 cells was therefore addressed in the present study. The intracellular distribution of GHR immunoreactivity within these clonal cells was also determined, since they also differ from normal pituitary cells in lacking secretory granules and having a much higher nuclear/cytoplasmic volume (Gourdji *et al.*, 1972). A summary of these results was recently reported (Hull *et al.*, 1994).

3.2. MATERIALS AND METHODS

A. Tissue Culture

GH_3 cells were obtained commercially (American Type Culture Collection, Bethesda, MD), and were cultured in Hams F-10 medium containing 12.5% horse serum and 2.5% fetal calf serum (Gibco BRL, Burlington, Ontario). Confluent cells (25 million) were washed in Hank's medium (containing 1% PMSF) (Gibco) to remove serum-containing culture medium prior to trypsinization and sedimentation. The cell pellets were then washed twice in serum-free medium prior to GHR analysis.

B. Northern Blotting

Total RNA was extracted from the GH_3 cell pellet by resuspension in 5.5 M guanidinium thiocyanate, containing 25 mmol sodium citrate/l, 0.2% (w/v) ß-mercaptoethanol and 0.5% (w/v) sodium lauryl sarcosine (pH 7.0). Total RNA was collected after centrifugation (125,000 x g, at 21°C for 22 h) through a bed of cesium trifluoroacetic acid (Pharmacia Fine Chemicals, Uppsala, Sweden, density 2.01) containing 100 mmol EDTA/l, pH 7.0 (Okayama *et al.*, 1987). RNA was assessed for degradation by electrophoresis and quantified by fluorimetry. Total RNA (10 µg, liver; 30-50 µg, pituitary) was electrophoresed in 1% agarose-formaldehyde gels and transferred to a nylon membrane (Zetaprobe, Millipore). A cDNA fragment encoding the majority of the extracellular domain of the rat GHR (pRat1-20) (American Cyanamid) (Figure 3.1) was gel-purified and labelled with ^{32}P-dCTP (New England Nuclear (NEN), Boston, MA) by random priming. The cDNA probe was then hybridized with the immobilized RNA in 30% (w/v) formamide (containing 0.75 mol NaCl/l, 25 mmol PIPES/l and 25 mmol EDTA/l, 0.2% (w/v) sodium dodecyl sulphate (SDS), 1x Denhart's reagent (0.1% (w/v) ficoll, 0.1% (w/v) BSA, 0.1% (w/v) polyvinylpyrrolidine), 100 µg salmon sperm DNA/l, pH 6.8) for 12 h at 42°C, following a three h incubation in the absence of the probe. Following a brief rinse in 2x SSC, the nylon membranes were washed with vigorous shaking at room temperature in 1% (w/v) SDS containing 2x SSC. Additional washes were performed at 42°C in 1% (w/v) SDS containing 1x SSC and subsequently in 1% SDS containing 0.5x SSC (1x SSC = 15 mmol sodium citrate/l and 150 mmol NaCl/l, pH 7.2). Membranes were then placed between intensifying screens and exposed to Kodak X-OMAT-AR film (Kodak, Rochester, New York, USA) for 7-14 d. The sizes of the RNA moieties hybridizing to the probe were determined by comparison with electrophoretically separable size markers (Boehringer Mannheim, Dorval, Quebec, Canada). The quantity of GHR mRNA in each hybridizing moiety was compared by laser densitometry of the autoradiographs.

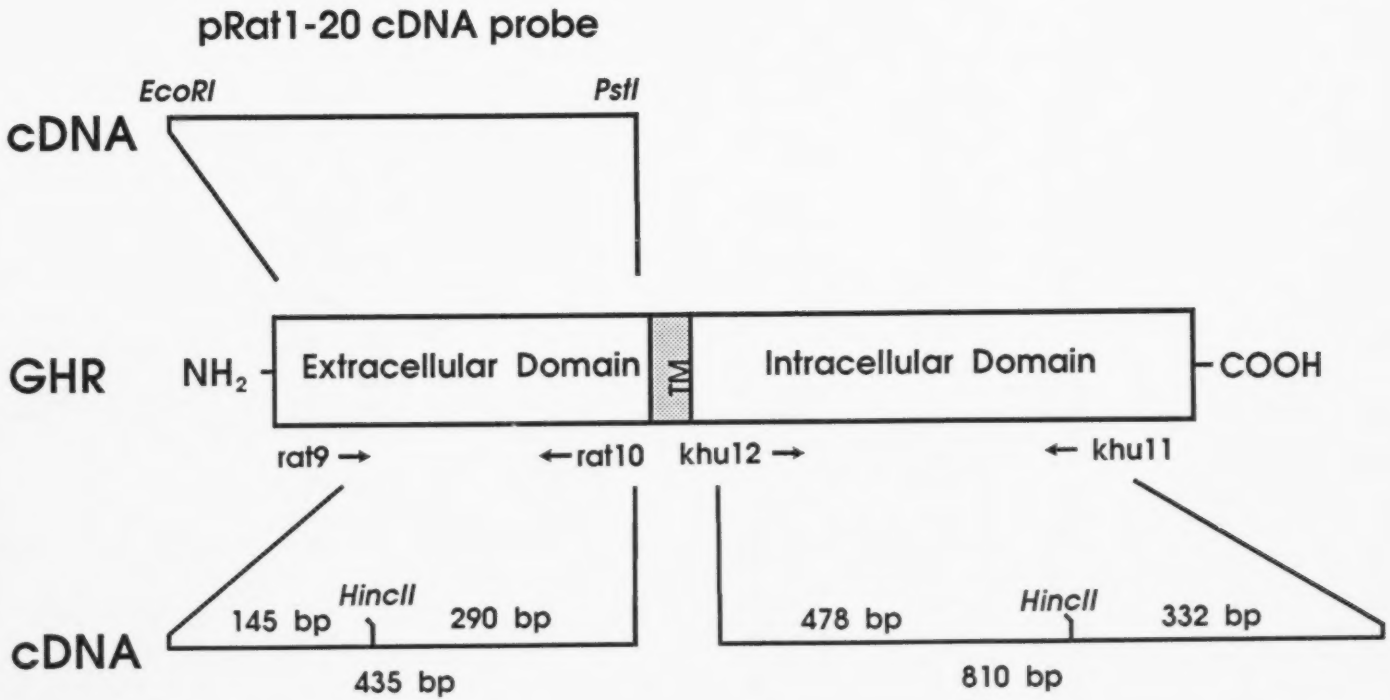

Figure 3.1. Sites of primer annealing and restriction enzyme digestion on the GHR cDNA (TM = transmembrane). The portion of the coding region for the extracellular domain contained within the cDNA probe "pRat1-20" is also shown.

D. Polymerase Chain Reaction

Total GH_3 RNA was also reverse transcribed by Superscript (100 U, BRL) in the presence of 100 pmol of oligodeoxythymine primer (Boehringer Mannheim), excess nucleotides (10 mmoles/l each of dCTP, dTTP, dATP and dGTP Boehringer Mannheim) and 5x H-RT buffer (BRL). The reactions were diluted with double distilled water (500:1 vol/vol) and an aliquot of each (5% of total volume) was added to a polymerase chain reaction (PCR) cocktail containing 5' -oligomer cGHR sense (CTGCGGCCGCAGGACCAGTTCCAAAGATTAA) and 3' antisense (AAGCGGCCGCGGCAGTAGTGGTAAGGCTTTC)oligonucleotides, deoxynucleotides (1.25 mmol/l of each), 1x PCR reaction buffer (50 mmol KCl/l, 10 mmol Tris-Cl/l, pH 8.4, 1.5 mmol $MgCl_2$/l, and 20 ug/ml gelatin) and Thermus aquaticus (Taq) DNA polymerase (5 U, BRL) (Kawasaki, 1990). The primers were based on the known sequence of a portion of the rat GHR transcript coding for the intracellular domain (Baumbach *et al.* 1989)(Figure 3.1). The reverse transcribed RNA was also amplified in the presence of 5' sense (CCTCCATTTGGATACCCTACTGCATTAAGC) and 3' antisense (GCATCACTGCTACTCCAAATATTCCAAAG) primers coding for a portion of the extracellular domain of the receptor. The mixture was overlayed with mineral oil (vol/vol), and heat denatured at 94°C for 3 min before 30 cycles of annealing (at 45°C for 1 min), extension (at 72°C for 3 min) and denaturing (at 94°C for 1 min) in a genetic thermal cycler (Minicycler, Fisher Scientific, Edmonton, Alberta). The PCR products were also digested for 2h with *Hinc*II for known cleavage sites in the regions coding for the extracellular and intracellular domains of the receptor. The PCR and digestion products were electrophoresed in 1.4% (w/v) agarose ethidium bromide-stained minigels and compared with HaeIII digested pUC 18 or Eco RI/BamHI digested lambda size markers.

The identity of the amplified PCR fragments was further elucidated by Southern blotting. Both intracellular and extracellular cDNA fragments were electrophoresed in 1.4% (w/v) agarose gels and transferred to Hybond N (Amersham, Oakville, Ontario) nylon membranes by capillary transfer (Turboblotter, Schleicher and Schuell,Keene, New Hampshire). Non-specific binding sites on the membrane were blocked by pre-

hybridization in 30% (w/v) formamide (containing 0.75 mol NaCl/l, 25 mmol PIPES/l and 25mmol EDTA/l, 0.2% (w/v) sodium dodecyl sulphate (SDS), 1x Denhart's Reagent (0.1% (w/v) ficoll, 0.1% (w/v) BSA, 0.1% (w/v) polyvinylpyrrolidine), 100 ug salmon sperm DNA/l, pH 6.8) for 1 hour. The immobilized cDNA was then hybridized with the pRat1-20 cDNA probe (Figure 3.1) in the same buffer for 12-18 h at 42°C. Following a brief rinse in 2x SSC, the nylon membranes were washed at room temperature in 0.1% (w/v) SDS containing 2x SSC. Additional washes were performed at 42°C in 1% (w/v) SDS containing 0.2x SSC (15 min) and subsequently in 1% (w/v) SDS containing 0.1x SSC (15 min)(1x SSC = 15 mmol sodium citrate/l and 150 mmol NaCl/l, pH 7.2). Membranes were then exposed to Reflection autoradiography film (NEN) in the presence of a Reflection intensifying screen (NEN) for 2 h at -70°C.

E. Western Analysis

GH_3 cells (approximately 25 million) were also homogenized in 1% (w/v) sodium dodecyl sulfate (SDS) containing 1 mM PMSF (Sigma, St.Louis, MO) and 10 µg/ml aprotinin (Sigma). Aliquots were subjected to electrophoresis in 15% acrylamide -SDS gels and subsequently electroblotted onto Immobilon P membranes (Millipore, Mississauga, Ont.) in 10 mM 3-[cyclohexylamino]-1-propane-sulfonic acid buffer (Sigma), pH 10, containing 10% methanol. The filter was incubated overnight with a polyclonal antibody raised against the recombinant rat GHBP (p(Ab)2,1:2000, Sadeghi *et al.*, 1990) in tris-buffered saline (TBS) (10 mM Tris (pH 7.5), 150 mM NaCl) containing 5% non-fat dried milk following a 1 h blocking step in the same buffer. Replicate blots were incubated in the absence of primary antibody or with antibody that had been pre-absorbed with 100 µg recombinant rat GHBP for 1 h. The blot was subsequently washed in TBS (4 x 30 min) and incubated with horseradish peroxidase-labelled goat anti-rabbit IgG (Amersham, Lancashire, England) for 1 h at a dilution of 1:1500. Following four 30 min washes in TBS containing 1% bovine serum albumin (BSA, Sigma), the filter was developed using a ECL kit (Amersham) and autoradiographed.

F. Radioimmunoassay

A heterologous system was first developed using a polyclonal antibody raised against rat GHBP (p(Ab)1, Sadeghi *et al.*, 1990) as the primary antibody and human GHBP (hGHBP, kindly donated by Dr. J.A. Wells, Genetech, San Francisco) as the standard and chloramine T-radioiodinated tracer. The tracer had a specific activity of approximately 40 μCi/ug and was > 90.0% precipitable with excess primary antibody, which specifically bound 26.7% of the tracer when present at a final dilution of 1:3000. Non-specific binding was approximately 3%. Tubes containing GH_3 cell homogenates or incubation media (1:1 - 1:8) were incubated with approximately 10 000 cpm of the tracer and poly(Ab)1 for 24 h at 4°C prior to the addition (at a final concentration of 1:120) of the goat anti-rabbit secondary antibody (Sigma). After a further 24 h incubation at 4°C, bound and free radioactivity were separated by centrifugation and aspiration of the supernatants. The precipitation of bound radioactivity was assisted by the addition of 100 ul protein A (Tachisorb, Calbiochem Corporation, La Jolla, CA), at a final concentration of 1:120. The hGHBP standard (over the range 2500 - 2.4 μg/l) and GH_3 cell homogenates were diluted in 0.05 sodium phosphate buffer (containing 1% bovine serum albumin), pH 7.0, in a sample volume of 200 μl.

For comparative purposes the GH_3 samples were similarly assayed in homologous radioimmunoassays for rGHBP (American Cyanamid), using p(Ab)1 or MAb 4.3 as the primary antibodies (at final dilutions of 1:4000 and 1:6000, respectively).

G. GHBP Release

The possibility that GHBP may be released from GH_3 cells and other rat tissues was also investigated by radioimmunoassay. Small fragments (approximately 3-5 mm^2) of fat, lung, brain, bladder, intestine, muscle, heart, spleen and liver, and entire pituitary glands were quickly excised from Sprague Dawley rats (Health Sciences Laboratory Animal Services (HSLAS)) and incubated for 1 h in Medium 199 (M199). Harvested GH_3 cells were also isolated and resuspended (3 million/ml) in M199. The media were then aspirated and replaced with fresh M199. Following a 5 h incubation, the media was aspirated and the cells dissolved in phosphate-buffered saline by Polytron homogenization

(tissues) or by sonification (GH_3 cells). The media and cell homogenates were concentrated in a Speedvac and assayed for GHBP content by the RIA systems described above, and the data was expressed relative to protein content, measured by the Bradford method (Bradford, 1976).

H. Immunogold Cytochemistry

GH_3 cells were pelleted in serum-free media, washed with PBS and fixed in 2% glutaraldehyde; 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min at room temperature. Samples were then prepared for cytochemistry as previously described (Harvey *et al.*, 1993). Briefly, the cell pellet was dehydrated in graded series of ethanols at -20°C prior to embedding in LR gold resin. This was polymerized in gelatin capsules under a UV lamp (360 nm) for 24 h. Ultrathin sections were cut and mounted on uncoated nickel grids.

For cytochemical staining, grids were floated on a drop of filtered 1% BSA for 10 min to block non-specific binding sites and transferred to a drop of primary antibody (MAb 263, raised against the rat GHR, Agen; MAb 4.3, raised against the rat hydrophilic GHBP tail, Sadeghi *et al.*, 1990) for 2 h at room temperature. Control grids were floated on drops of PBS or of an unrelated monoclonal antibody raised against *Brucella* (Agen). Following washes with PBS, grids were floated on drops of a colloidal gold-labelled goat anti-mouse secondary antibody (1:10 in PBS) and washed in PBS and then water. Grids were stained with uranyl acetate and lead citrate to enable recognition of ultrastructural morphology.

The density of gold particle staining in photographs (magnification x7400) was also compared in GH_3 cells and normal rat pituitary cells. The number of gold particles was counted in 12 separate 1 cm^2 boxes in nuclear and cytoplasmic compartments of GH_3 cells stained with MAb 4.3 (n=3) and MAb 263 (n=1). Nuclear/cytoplasmic ratios were also determined for pituitary cells stained with these antibodies (as described in Harvey *et al.* 1993) for comparative purposes. As secretory granules are not present in GH_3 cells, the cytoplasm of pituitary sections was divided into secretory granule and non-secretory granule compartments.

3.3 RESULTS

A. Northern Analysis

As expected, RNA moities of 4.4 kb and 1.2 kb (in a ratio of approximately 1:1.7) in rat liver hybridized with the rat GHR cDNA probe (Figure 3.2). A 4.4 kb moiety was also observed in GH_3 cells, although of much lower abundance. A 1.2 kb transcript was not evident, however, even when the blot was overexposed or when the gels were overloaded with a 5-fold excess of total RNA.

B. Polymerase Chain Reaction

After 30 cycles of amplification by PCR with oligonucleotide primers for the extracellular (rat 9 and 10) or intracellular (khu 11 and 12) coding regions of the GHR, cDNA fragments of predicted size (435 bp and 800 bp, respectively) were generated from reverse transcribed GH_3 RNA, identical to those generated from liver RNA (Figure 3.3). A second cycle of amplification followed by endonuclease digestion of the GH_3 (Figure 3.3) and hepatic (data not shown) products with *Hinc*I resulted in smaller fragments of expected size (approximately 480 bp and 330 bp for intracellular cDNA, 150 and 300 for extracellular cDNA). The cDNA moiety amplified with khu9 and 10 hybridized with a cDNA probe coding for the extracellular domain, whereas hybridization did not occur with fragments generated by amplification with oligonucleotide primers for the intracellular domain (Figure 3.4).

C. Western Analysis

Translation of the GHR/GHBP gene transcripts was indicated by Western analysis. Proteins in GH_3 cells of 100 kDa and 46 kDa were immunoreactive with a polyclonal antibody (p(Ab)2) raised against the rat GHBP (Figure 3.5). These were of identical size to proteins in rat liver that were similarly immunoreactive. In both cases, the larger proteins were not labelled after the primary antibody was preabsorbed with recombinant rat GHBP, however, labelling of the 46 kDa proteins was unaltered or enhanced in the presence of rGHBP. Neither protein, however, was labelled in the absence of the primary antibody or when this was substituted with preimmune rabbit serum (data not shown).

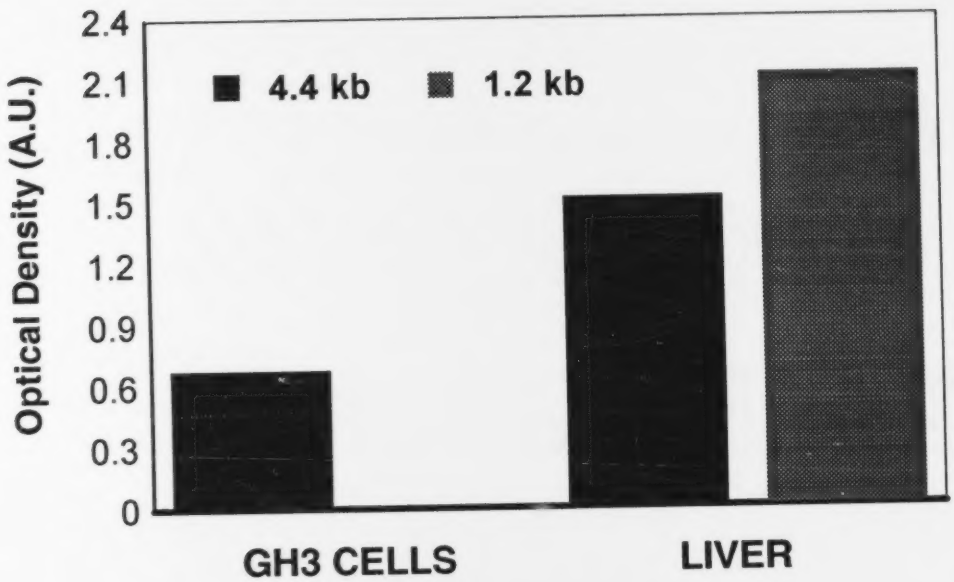

Figure 3.2. Graphical representation of Northern analysis of GH_3 and rat liver RNA. The membrane was incubated with a rat GHR cDNA probe, and the sizes of hybridizing moieties was determined by comparison with molecular size markers. The amount of hybridization to each moiety was determined by laser densitometry and expressed in arbitrary units (A.U.).

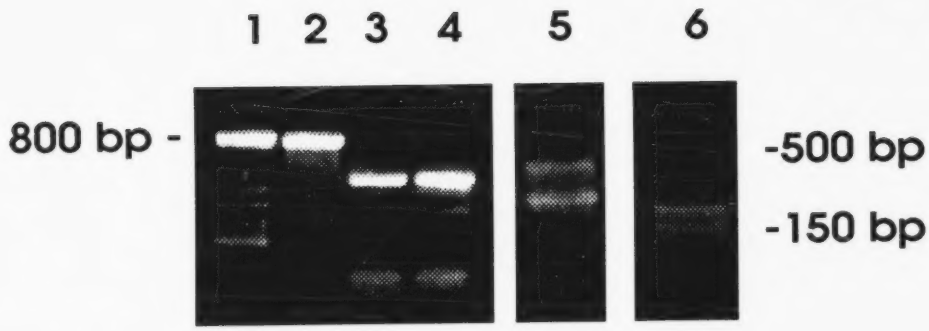

Figure 3.3. PCR analysis of GH3 cell cDNA. Reverse-transcribed total RNA extracted from rat liver (lane 1) and GH3 cells (lane 2) was amplified by the polymerase chain reaction with 3' and 5' oligonucleotide primers for the intracellular domain of the rat liver GHR cDNA (khu 11 and 12). HincII digests of the PCR products derived from GH3 cDNA samples are also shown (lane 5). The hepatic (lane 3) and GH3 cell (lane 4) cDNA was also amplified in the presence of 3' and 5' primers for the extracellular domain (rat 9 and rat 10) and digested by Hinc II (shown in lane 6 for GH3 cDNA). The cDNA fragments were subjected to 1.4% agarose gel electrophoresis and were visualized by ethidium bromide. When Superscript was omitted from the original reverse transcription mixture, no amplification was observed with either set of primers (data not shown).

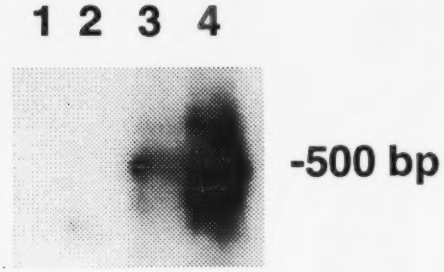

Figure 3.4. Southern analysis of cDNA fragments generated by the polymerase chain reaction. Moities amplified by intracellular primers from GH3 cell (lane 1) and rat liver (lane 2) cDNA were subjected to electrophoresis, transferred to nylon membranes and hybridized with radiolabelled pRat1-20, which encodes the extracellular domain of the rat GHR. GH3 (lane 3) and hepatic (lane 4) cDNA amplified in the presence of extracellular primers was similarly screened for homology with extracellular GHR sequences. As expected, specific hybridization was observed with fragments amplified with extracellular, but not intracellular primers.

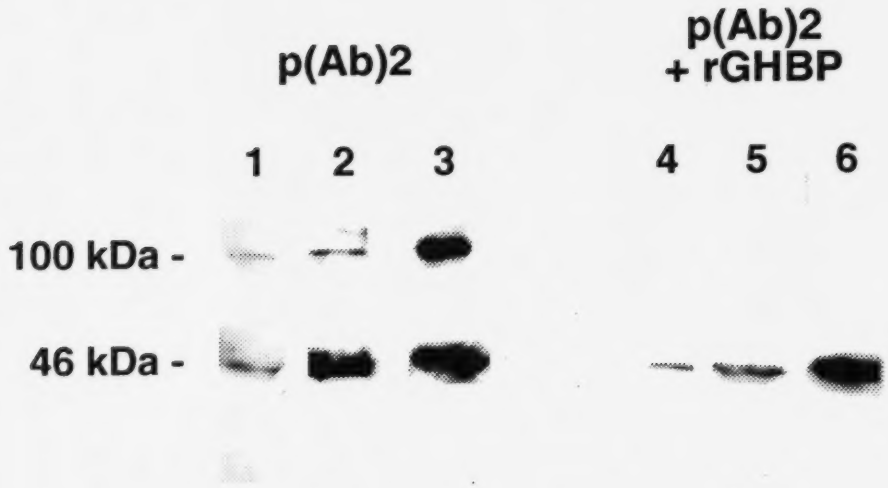

Figure 3.5. Western analysis showing proteins immunoreactive with p(Ab)2 antisera (raised in rabbits against recombinant rat GHBP (rGHBP)) in GH3 cells (lanes 1 and 2) and rat liver (lane 3). A replicate blot containing GH3 cell (lanes 4 and 5) and rat liver (lane 6) homogenates was also stained with p(Ab)2 that had been preabsorbed with rGHBP.

D. Immunogold Cytochemistry

Immunoreactivity for the GHR/GHBP was also evident in GH_3 cells immunocytochemically stained with MAb 263 (Figure 3.6b). Immunoreactive gold particles were found throughout the cytoplasm and in the nucleus. The nuclear:cytoplasmic ratio in gold particle abundance (grains/cm^2 photograph) was 1:1.8. Immunocytochemical staining of nuclear and cytoplasmic compartments (in a ratio of 1:1.72) was also shown using MAb 4.3 (raised against the rat GHBP hydrophilic tail) as the primary antibody (Figure 3.6a). In both cases staining was restricted to intracellular compartments and was not observed extracellularly. The specificity of this staining was indicated by its absence when MAb 263 or MAb 4.3 were replaced by PBS or unrelated antibodies (data not shown).

E. Radioimmunoassay

GHRs and/or GHBPs in GH_3 cells were also detected by radioimmunoassay. Cellular extracts and incubation media inhibited the binding of ^{125}I-hGHBP or ^{125}I-rGHBP to p(Ab)1 or MAb 4.3 in a manner parallel with the hGHBP and rGHBP standards (Figure 3.7). The amount of hGHBP/rGHBP immunoreactivity in GH_3 cells was, however, far less than that in the pituitary gland or in any extrapituitary tissue (Figure 3.8). Estimates of rGHBP immunoreactivity in GH_3 cells and tissue extracts using p(Ab)1 were generally comparable with p(Ab)1 immunoreactive hGHBP estimates ($r=.957$, $p,<0.001$, $n=11$) and hGHBP estimates are therefore not shown in Figures 3.8 and 3.9. When GH_3 cells and rat tissues were incubated in Medium 199, GHBP immunoreactivity was measurable in each incubation media, although at concentrations far less (mean = 1.7 $\pm$ 0.3%) than those in tissues (Figure 3.8). Hepatic media contained 1.88% of the GHBP in tissue whereas pituitary media contained 3.13% of the pituitary GHBP content. In contrast, the culture media of GH_3 cells contained 5.81% of the tissue GHBP content.

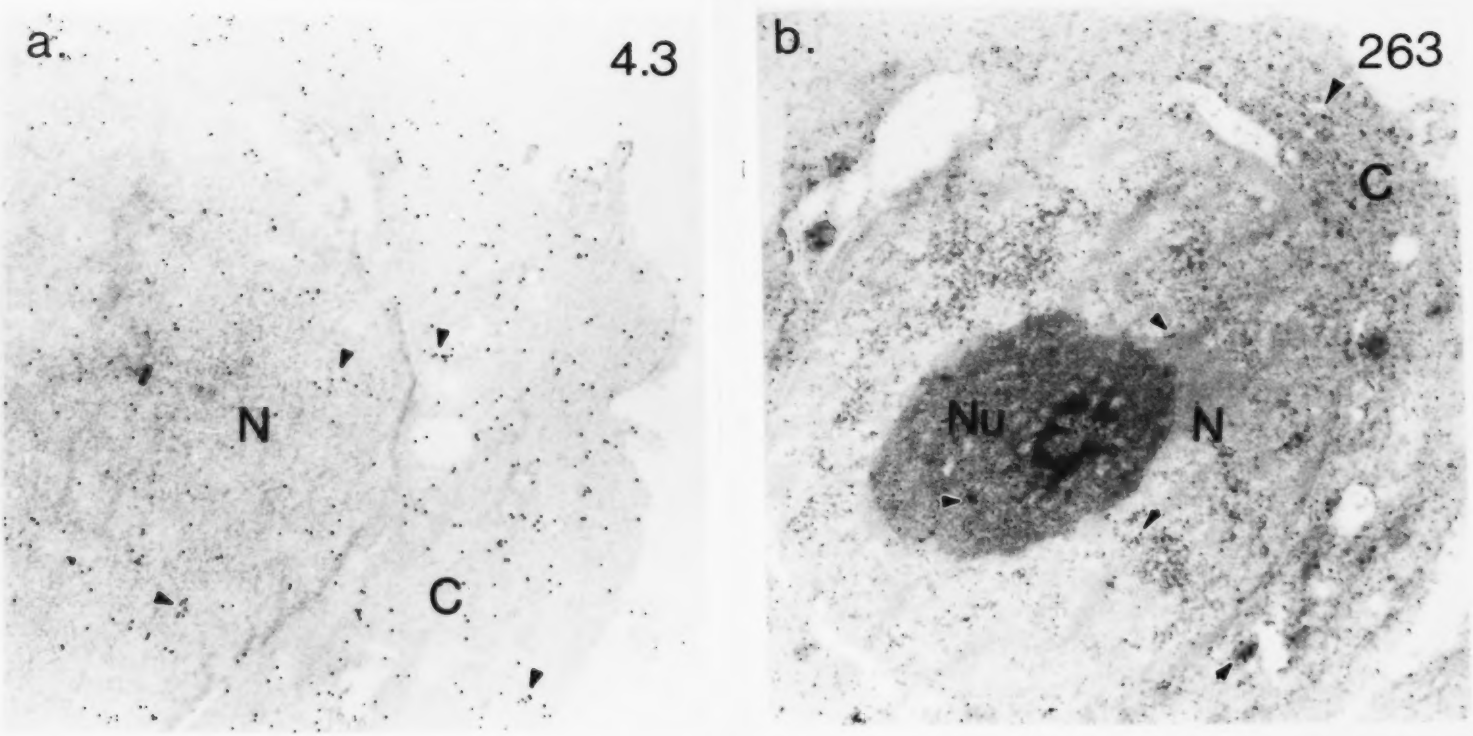

Figure 3.6. Immunocytochemical staining of GH3 cells. Pelleted cells were fixed in 2% glutaraldehyde/0.2% paraformaldehyde, dehydrated and embedded in LR gold resin. Ultrathin sections were prepared on uncoated nickel grids, and were stained with either MAb 4.3 (panel a), raised against the hydrophilic tail of the rat GHBP, or MAb 263 (panel b), which recognizes an epitope in the GH-binding domain of the rat GHBP. GHBP-like immunoreactivity was detected by colloidal gold-labelled anti-mouse IgG antibody and cellular ultrastructure was revealed by staining with uranyl acetate and lead citrate. Abbreviations; Nu, nucleolus; N, nucleus; C, cytoplasm.

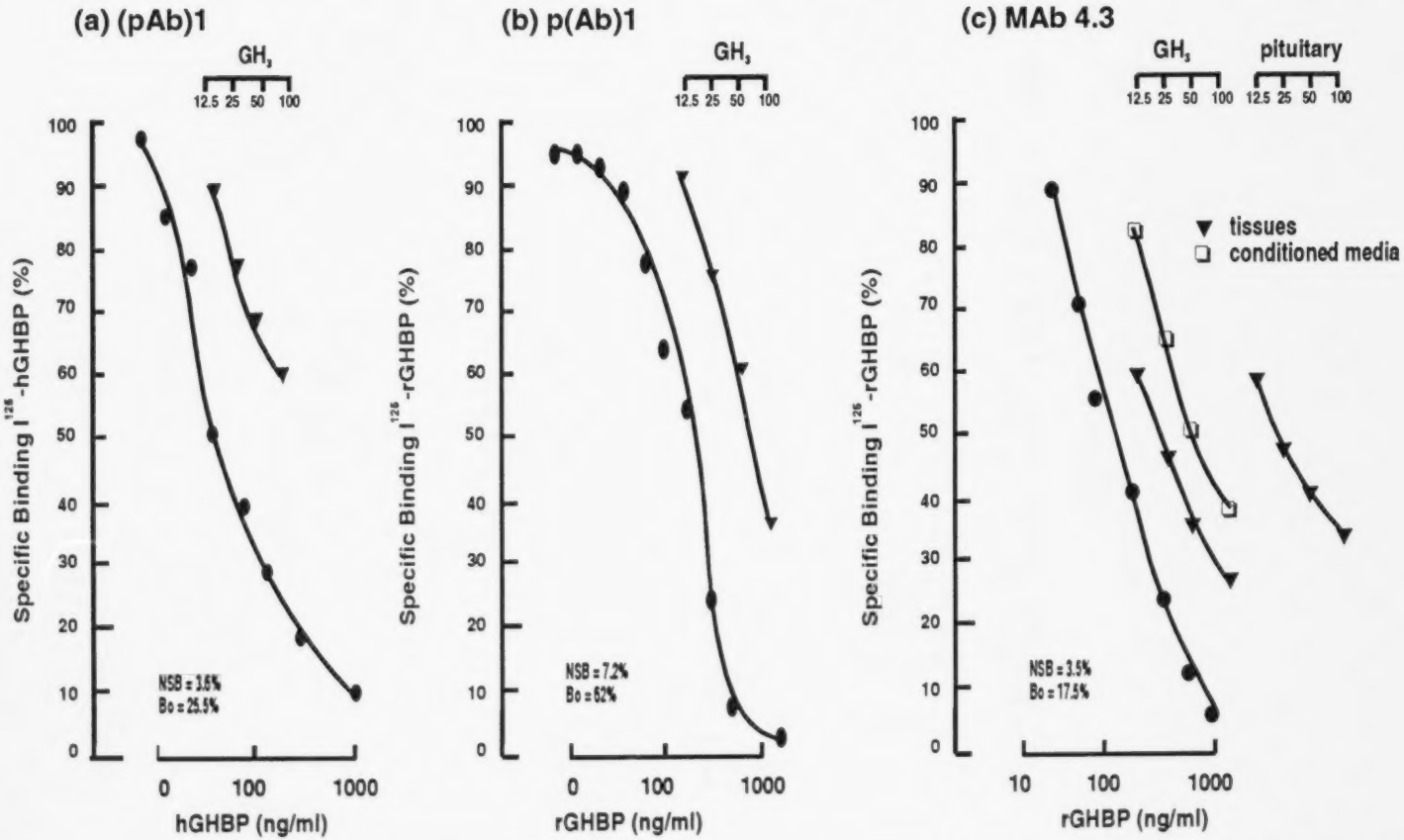

Figure 3.7. Cross-reactivity of GH_3 cells and GH_3-conditioned media with (b) p(Ab)1 and (c) MAb 4.3 in a radioimmunoassay using rGHBP as the standard and I^{125}-labelled tracer. (a) p(Ab)1 was also employed in a radioimmunoassay using hGHBP as the standard and I^{125}-labelled tracer. GH3 cell-conditioned media did not, however, signficantly displace I^{125}-labelled rGHBP binding to p(Ab)1. The cross-reactivity of rat pituitary extracts with MAb 4.3 is shown for comparison. Levels of total binding (Bo) and non-specific binding (NSB) are indicated for each assay.

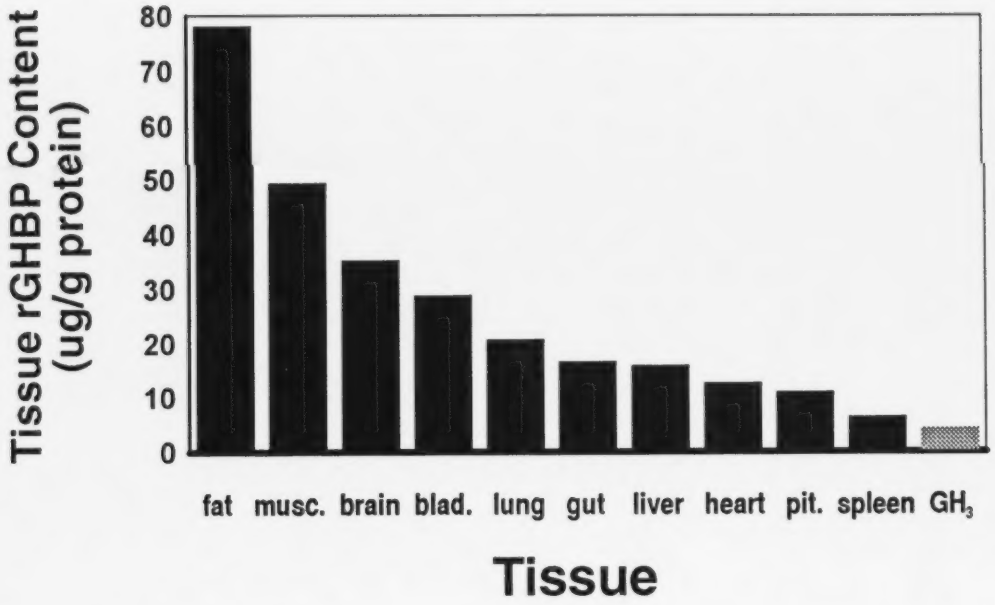

Figure 3.8. Immunoreactive GHBP concentrations (ug/g tissue protein) in rat tissues and GH_3 cells. Abbrevations: musc., muscle; blad., bladder; pit., pituitary gland.

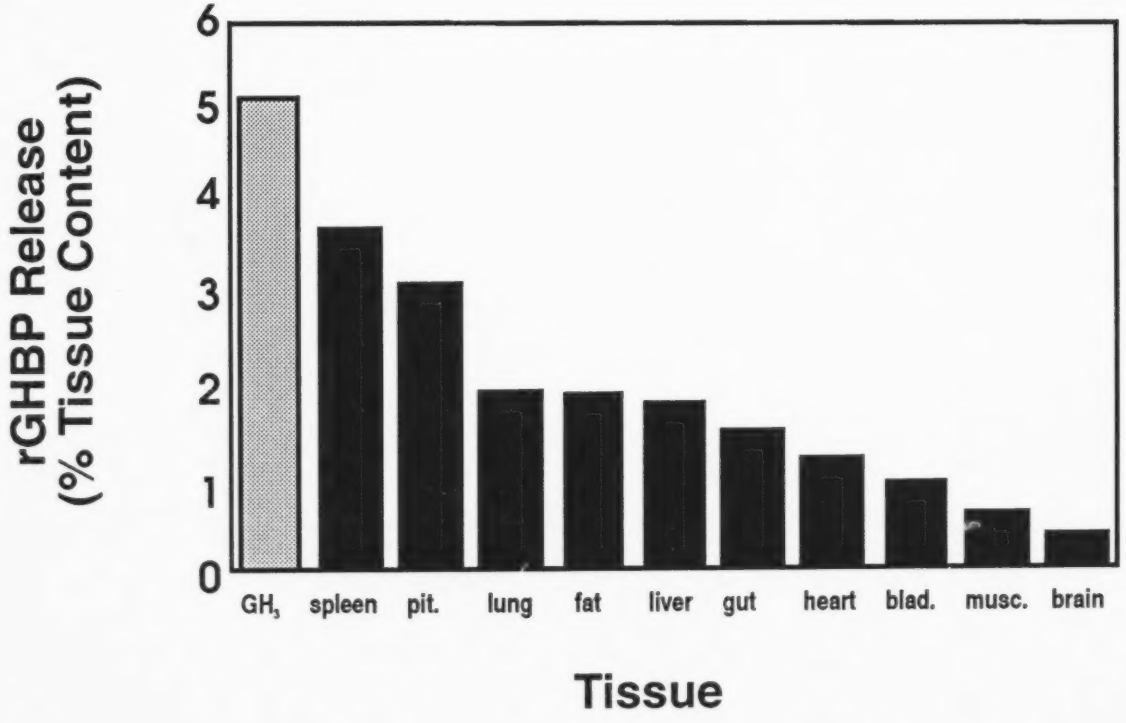

Figure 3.9. Release of immunoreactive GHBP from rat tissues and GH_3 cells during a 5h incubation period, expressed relative to tissue GHBP concentration.

3.4. DISCUSSION

Tumorous somatolactotrophs differ from normal adenohypophyseal cells in having an overabundance or deficiency of functional receptors for many stimulatory or inhibitory factors regulating GH and prolactin release (Gourdji *et al.*, 1982). Despite these receptor anomalies, the results of this study clearly demonstrate normal expression of the GHR gene in GH_3 cells, in which immunoreactive GHRs and/or GHBPs are ubiquitously distributed.

Expression of the GHR gene in GH_3 cells may, however, be less than in liver and other GH target sites, since GHR gene transcripts were not easily detected by Northern blotting. Moreover, while the ratio of GHBP:GHR transcripts in the rat liver is >7:1 (Bingham *et al.*, 1994), Northern analysis failed to detect GHBP RNA in GH_3 cells. This is, however, similar to normal pituitary cells, in which Bingham *et al.* (1994) found the abundance of GHR mRNA to be five-fold greater than GHBP transcripts. The immunoreactive GHBP content in GH_3 cells was also much lower than in the rat pituitary gland and in other extrapituitary tissues, consistent with a reduced rate of GHR gene expression. This reduced GHBP concentration may thus facilitate receptor-mediated GH action and provide an explanation for the autoregulatory mechanism in tumorous somatotrophs that suppresses GH secretion. The presence of membrane binding sites for GH, linked to intracellular signal transduction systems, was not, however, directly assessed.

Although the intracellular GHBP content of GH_3 cells is far less than that in hepatic and extrahepatic rat tissues, the extracellular:intracellular GHBP ratio is far higher for incubated GH_3 cells than for incubated rat tissues (Figure 3.8). The constitutive release of GHBP from these cells may thus occur at a faster rate than in rat tissues that possess (eg. pituitary and brain) or lack (eg. liver and muscle) secretory granules. Thus, although the steady-state levels of GHR gene transcripts are lower than in normal rat tissues, their turnover rate may be accelerated in these tumorous cells. Moreover, since the intracellular content in GH_3 cells is less than in normal tissues, the rate of GHBP release may also be accelerated in these cells. This possibility is also supported by the demonstration that GH abundance is far lower in GH_3 cells than in rat somatotrophs but

the constitutive rate of GH release is much greater from GH_3 cells, which have extracellular:intracellular GH ratios of 2:1 (Tixier-Vidal *et al.*, 1980).

GHBP has previously been shown to be constitutively released from hepatocytes (Amit *et al.*, 1994, Barnard *et al.* 1994) but not from adipocytes (Frick *et al.*, 1994) or trophoblasts (Barnard *et al.*, 1994) that possess intracellular GHBP immunoreactivity. This therefore contrasts with our finding of GHBP immunoreactivity in the media of all the tissue explants incubated. The finding that media GHBP immunoreactivity was greater for several extrahepatic tissues than for liver explants was also unexpected, as this may indicate that numerous tissues contribute to circulating GHBP concentrations (Harvey& Hull, 1994). Nevertheless, because of its size, the liver would still probably be of primary importance in regulating plasma GHBP concentrations, even if other tissues contributed to an intravascular pool. It is, however, also possible that the presence of GHBP immunoreactivity in the media of all cultured tissues may merely reflect non-specific release of GHBP from damaged cells or the shedding of surface GHRs by tissue protease activity, especially as the amount of media GHBP was <5% of the tissue GHBP content. This possibility awaits investigation.

The subcellular distribution of GHR/GHBP immunoreactivity in GH_3 cells included both cytoplasmic and nuclear compartments. This is therefore similar to GHR/GHBP distribution in normal pituitary cells, hepatocytes, neurons and glia (Lobie *et al.*, 1991; Fraser& Harvey, 1992; Lobie *et al.*, 1992; Lobie *et al.*, 1993). Indeed, the ratio between nuclear and cytoplasmic staining by MAb 263 in GH_3 cells (1:1.8) was comparable to that in pituitary cells which contain secretory granules (1:1.65, calculated on figures presented by Harvey *et al.* 1993). Most of the GHR/GHBP immunoreactivity in normal pituitary cells is, however, in the secretory granules, as the nuclear:cytoplasmic GHBP ratio falls to 1:0.85 in secretory granule-free cytoplasm. Harvey *et al.* (1993) found no MAb 4.3 GHBP immunoreactivity in normal pituitary cells. The occurrence of MAb 4.3 immunoreactivity in the nuclei of GH_3 cells therefore differs from normal pituitary cells and is more akin to the GHBP distribution in more traditional sites of GH action. Lobie *et al.* (1991), for instance, found significant MAb 4.3 GHBP immunoreactivity in hepatic nuclei. It is, however, unclear if nuclear GHBPs have direct or indirect effects on gene

transcription within the nucleus.

In summary, these results demonstrate that GHR expression in GH3 cells is comparable with that in normal pituitary cells. These tumorous somatolactotrophs could thus provide a potential model for studies on GHR/GHBP synthesis and release and for studying GH effects on pituitary function. The release of GHBP from pituitary cells may also indicate, for the first time, an extra-hepatic source of the GHBP in plasma.

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CHAPTER 4: THYROID GLANDS: NOVEL SITES OF GROWTH HORMONE ACTION?[1]

4.1. INTRODUCTION

Interactions between growth hormone (GH) and thyroid hormones are well-documented, as these factors act synergistically and additively to affect growth and development of both peripheral and central tissues (McNabb & King, 1993). These hormones also appear to be reciprocally regulated in a species-specific manner. For instance, thyroid hormones stimulate GH synthesis in mammals via thyroid hormone-response elements in the GH gene (Iranmanesh *et al.*, 1991; Valcavi *et al.*, 1992; Rodriguez-Arnao *et al.*, 1993), whereas triiodothyronine (T_3) and thyroxine (T_4) inhibit *in vivo* GH synthesis and release and *in vitro* thyroid hormone releasing hormone (TRH)/growth hormone releasing factor (GRF)-stimulated GH release in birds (Harvey *et al.*, 1990; Harvey, 1990a; Harvey, 1990b; Denver & Harvey, 1991; Harvey *et al.*, 1991). This inhibitory effect is thought to be mediated at the hypothalamic level by reduced TRH secretion and (for T_3) at the pituitary level by reduced TRH receptors (Harvey *et al.*, 1991). Conversely, the effects of GH on the thyroid axis appear to be relatively consistent between species, as GH lowers circulating thyroxine concentrations in both mammals and birds (Decuypere & Kuhn, 1988; Darras *et al.*, 1992; Darras *et al.*, 1993) and can induce central hypothyroidism (via suppression of TRH-induced thyroid stimulating hormone (TSH) secretion) in GH-deficient men and in untreated acromegalics (Porter *et al.*, 1973; Lippe *et al.*, 1975; Grunfeld *et al.*, 1988; Miyakawa *et al.*, 1988). However, *in vivo*, GH is a potent enhancer of thyroid function, as it increases plasma concentrations of T_3 by enhancing 5'monodeiodinase activity (Bjorn-Hansen *et al.*, 1991; Darras *et al.*, 1991; Geelhoed-Duijvestijn *et al.*, 1992) and/or by inhibiting the conversion of T_3 into the inactive metabolite diiodothyronine (T_2) (Darras *et al.*, 1992; Darras *et al.*,

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1993). Thus, the inhibitory effect of GH on plasma T_4 is mediated in part by enhanced conversion to T_3. It is, however, uncertain if GH directly affects thyroid hormone secretion. This possibility has therefore been examined in the present study.

4.2. MATERIALS AND METHODS

A. GH effects on thyroid function

(i) ***In vivo:*** The effect of chronic *in vivo* GH treatment on *in vitro* thyroidal T_4 release was investigated in 4-week male Leghorn chickens intramuscularly injected with 1-100 µg recombinant chicken GH (rcGH)/d for 14 d. The birds were sacrificed by decapitation 2 h subsequent to the last injection, and the thyroid glands rapidly dissected out and collected into ice-cold medium M199 (Gibco, Grand Island, New York). The glands were individually pre-incubated in a shaking water bath at 39°C for 1 h. The medium was then discarded and replaced by fresh medium in the presence or absence of 200 mU (100 µg/ml) of a crude preparation of bovine thyrotropin-stimulating hormone (bTSH) (Sigma Chemical Company, St. Louis, MO). Each tube was gassed with 95% O_2; 5% CO_2, capped, and incubated for a further 3 h, after which the medium was aspirated and stored at -20°C prior to analysis. Chicken thyroid glands remain viable under these conditions for at least 12 h and the basal release of T_4 is responsive to this dose of TSH and suppressible by SRIF and media of low calcium concentration (Lam *et al.*, 1986; Lam *et al.*, 1987).

(ii) ***In vitro*:** Direct effects of GH on the release of T_4 was similarly determined using glands isolated from untreated birds. These tissues were incubated for 3 h in media containing various concentrations of chicken GH (0-10^{-5}mol GH/l) in the presence or absence of 200 mU (100 µg/ml) bTSH.

(iii) **Radioimmunoassay**: The concentrations of T_4 in the incubation media were measured by radioimmunoassay (RIA), using commercial reagents as previously described (Lam *et al.*, 1986; Lam *et al.*, 1987). T_4 release was corrected for tissue mass and expressed relative to the controls and analysed by analysis of variance (ANOVA).

B. GH-Binding Assay

GH-binding sites on thyroidal membranes were determined as described previously for hepatic membranes (Vanderpooten *et al.*, 1991). Briefly, thyroid glands were dissected from adult Leghorn chickens, quick-frozen in liquid nitrogen and stored at -70° C. The tissue was homogenized in ice-cold 20 mM phosphate buffer (pH 7.4) and fractionated by differential centrifugation. The homogenate was centrifuged at 500 *g* for 10 min and the supernatant then centrifuged at 100 000 *g* for 45 min at 4° C. The 100 000 *g* pellet was then resuspended in 20 mmol/l ice-cold phosphate buffer (pH 7.4). Protein estimations were performed using the assay of Bradford. Following displacement of endogenous GH with $MgCl_2$, the membranes were incubated at room temperature for 20 h with 35 000 cpm ^{125}I-labelled cGH in the presence or absence of unlabelled cGH (500 ng/100µl per tube). The tracer was prepared by the chloramine T method and purified on Sephadex G-100 (Pharmacia Fine Chemicals AB, Uppsala, Sweden). The tracer had a specific activity of 40-80 µCi/µg and was > 95% precipitable with excess 10% (v/v) trichloroacetic acid. Following incubation with the tracer, 2 ml ice-cold assay buffer/tube was added and the bound and free radioactivity were separated after centrifugation for 10 min at 2000 *g* (4° C). Specific binding was calculated by subtracting non-specific binding (in the presence of excess unlabelled GH) from total binding. Competition displacement of tracer binding was determined in the presence of cGH at concentrations of 1-1000 nmol/l, while saturable binding was determined by the addition of 1 x 10^4 c.p.m. of the tracer. Data were analysed using LIGAND software (Biosoft, Elsevier, Cambridge, U.K.).

C. GH receptor mRNA

(i) Northern Blot Analysis: Chronic hyper- or hypothyroidism was induced in adult White Leghorn chickens by daily i.p. injections of 100 µg T_3/kg body weight or 50 µg thiourea/kg (respectively) for 13 d (n=12 for each group). Previous studies have established that these treatments are effective in inducing thyroid excess and deficiency syndromes (Harvey, 1983). Control birds were injected with an equal volume (1 ml/kg) of 0.9% (w/v) NaCl. Thyroid glands were rapidly dissected from the birds 2 h subsequent

to the last injection, frozen in liquid nitrogen and stored at -80°C. Total cellular RNA was extracted from pools of 3 thyroid glands in 5.5 M guanidinium thiocyanate, containing 25 mmol sodium citrate/l, 0.2% (w/v) ß-mercaptoethanol and 0.5% (w/v) sodium lauryl sarcosine (pH 7) and collected after centrifugation (125,000 x g, at 21°C for 22 h) through a bed of cesium trifluoroacetic acid (Pharmacia Fine Chemicals, Uppsala, Sweden, density 2.01) containing 100 mmol EDTA/l, pH 7.0 (Okayama *et al.*, 1987) The RNA was quantified spectrophotometrically and 15 µg aliquots were subjected to electrophoresis in 1% (w/v) agarose and 3.1% (w/v) formaldehyde gels and transferred to nylon membranes by capillarity. A rabbit GHR (rGHR) complementary RNA (cRNA) probe was constructed from a 638 base pair (bp) Bam H1/EcoR1 fragment (donated by Dr. W.I. Wood, Genetech, San Francisco, California,USA) of the rabbit hepatic GHR cDNA sequence coding for portions of the extracellular and transmembrane domains (Leung *et al.*, 1987). The plasmid pGEM3Z (Promega Corporation, Madison, Wisconsin, USA) containing this sequence was linearized by Bam H1 digestion and transcribed by T7 polymerase using a riboprobe kit (Promega Corporation) in the presence of [^{32}P]αCTP (800 uCi/mmol; New England Nuclear, Mississauga, Ontario, Canada). This probe specifically hybridizes with GHR RNA in chicken tissues (Fraser *et al.*, 1990). Transcription of this plasmid with SP6 polymerase (Promega Corporation) generates a sense RNA probe that does not hybridize with chicken mRNA (Fraser *et al.*, 1990). The cRNA probe was then hybridized with the immobilized RNA in 60% (w/v) formamide (containing 0.75 mol NaCl/l, 25 mmol PIPES/l and 25mmol EDTA/l, 0.2% (w/v) sodium dodecyl sulphate (SDS), 1x Denhart's Reagent (0.1% (w/v) ficoll, 0.1% (w/v) BSA, 0.1% (w/v) polyvinylpyrrolidine), 100 ug salmon sperm DNA/l, pH 6.8) for 12 h at 55°C, following a three h incubation in the absence of the probe. Following a brief rinse in 2x SSC, the nylon membranes were washed at room temperature in 0.1% (w/v) SDS containing 2x SSC and subsequently at 75°C in 1% (w/v) SDS containing 0.1x SSC (1x SSC = 15 mmol sodium citrate/l and 150 mmol NaCl/l, pH 7.2). Membranes were then placed between intensifying screens and exposed to Kodak X-OMAT-AR film (Kodak, Rochester, New York, USA) for 2 d. The sizes of the RNA moieties hybridizing to the probe were determined by comparison with electrophoretically separable size markers

(Boehringer Mannheim, Dorval, Quebec, Canada). The quantity of GHR mRNA in each sample was determined by laser densitometry of the autoradiographs and corrected for loading error by comparison with the hybridization of a gamma-actin mouse cRNA probe (generously provided by Dr. R. Chibbar, University of Alberta) to the same blot. GHR mRNA concentrations in the different treatment groups were compared by ANOVA.

(ii) Polymerase Chain Reaction: Total thyroid RNA was also reverse transcribed by Superscript (100 U, BRL) in the presence of 100 pmol of oligodeoxythymine primer (Boehringer Mannheim), excess nucleotides (10 mmoles/l each of dCTP, dTTP, dATP and dGTP Boehringer Mannheim) and 5x H-RT buffer (BRL). The reactions were diluted with double distilled water (500:1 vol/vol) and an aliquot of each (5% of total volume) was added to a polymerase chain reaction (PCR) cocktail containing 5' -oligomer cGHR sense (CCTCGATTTGGATACCATATTGTGTTAAGC) and 3'-oligomer rGHR antisense primers (CTGTTACGGCCAGCCCACACACTCCGAAG) (15 pmol of each) (Figure 4.1), deoxynucleotides (1.25 mmol/l of each), 1x PCR reaction buffer (50 mmol KCl/l, 10 mmol Tris-Cl/l, pH 8.4, 1.5 mmol $MgCl_2$/l, and 20 ug/ml gelatin) and Thermus aquaticus (Taq) DNA polymerase (5 U, BRL) (Kawasaki, 1990). The primers were based on the known sequence of a portion of the chicken GHR transcript coding for the extracellular domain (Burnside *et al.*, 1991). The reverse transcribed RNA was also amplified in the presence of 5` sense (CTGCGGCCGCAGGACCAGTTCCAAAGATTAA) and 3` antisense (AAGCGGCCGCGGCAGTAGTGGTAAGGCTTTC) oligonucleotides coding for a portion of the intracellular domain of the receptor (Figure 1) (Burnside *et al.*, 1991). The mixture was overlayed with mineral oil (vol/vol), and heat denatured at 94°C for 3 min before 30 cycles of annealing (at 50°C for 1 min), extension (at 72°C for 3 min) and denaturing (at 94°C for 1 min) in a genetic thermal cycler (Precision Instruments, Chicago, Illinois, USA). The PCR products were also digested for 2 h with restriction endonucleases (BamHI and NcoI or EcoRI and HaeIII for known cleavage sites in the regions coding, respectively, for the extracellular and intracellular domains of the receptor (Burnside *et al.*, 1991). The PCR and digestion products were electrophoresed in 1.4%

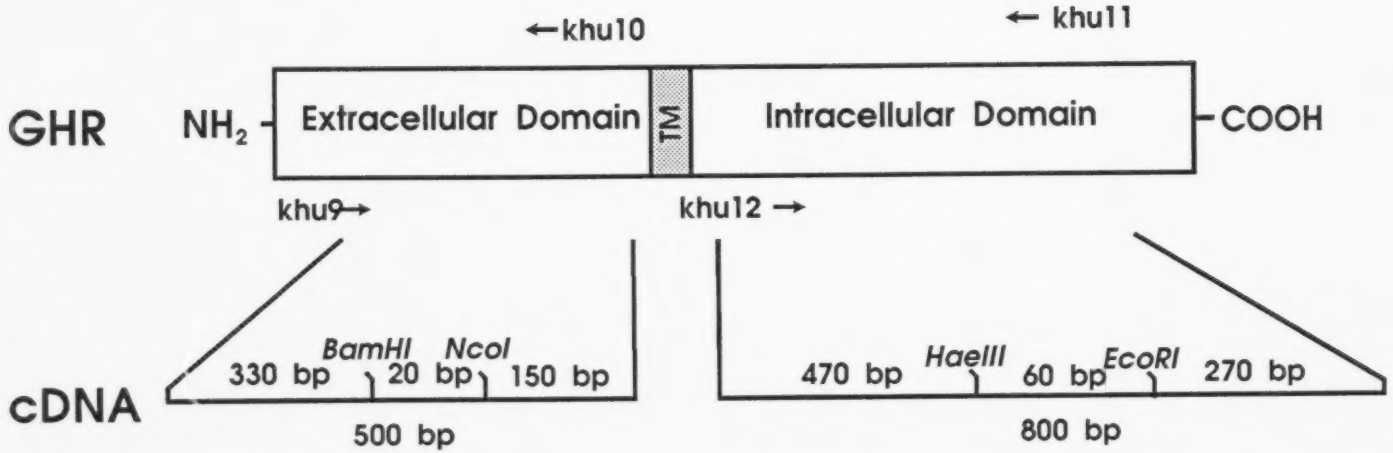

Figure 4.1. Sites of primer annealing and restriction enzyme digestion on the GHR cDNA (TM = transmembrane).

(w/v) agarose ethidium bromide-stained minigels and compared with HaeIII digested pUC 18 or Eco RI/BamHI digested lambda size markers.

D. GH Receptor Immunocytochemistry

Freshly collected thyroid glands from euthyroid birds were prepared for immunocytochemistry by overnight fixation in Bouin's Solution, after which the tissues were cryoprotected in 30% sucrose for approximately 4 h. Cryoprotected thyroid glands were cut into 10-16 um sections and thaw-mounted onto chrom-alum/gelatin-coated slides. The slides were washed in 0.1 mol PBS/l (pH 7.4) followed by a 30 min incubation in 0.1% (v/v) hydrogen peroxide to extinguish endogenous peroxidase activity. Non-specific binding was reduced by a 20 min pre-incubation in 5% (v/v) normal goat serum (NGS), GHR immunoreactivity was visualized by incubation in a rabbit anti-chicken growth hormone receptor antibody (1:250 in PBS containing 1% (v/v) NGS) for 12-24 h followed by biotinylated goat anti-rabbit antibody (2 h) and ABC-complex (1 h). The avidin-biotin reaction was visualized by incubation in 0.05% (w/v) DAB (Sigma) for 3 to 5 min. Slides were washed in several changes of 0.1 mol PBS/l between each step, except following the pre-incubation with 5% (v/v) NGS. The specificity of staining was demonstrated by replacement of the primary antibody with non-immune rabbit serum or PBS. The primary antibody was also pre-absorbed by a 2 h incubation with chicken liver membranes (an abundant source of GH receptors) in the presence of protease inhibitors (EDTA, 1 g/l; bacitracin, 30 mg/l; and apoprotinin, 30 µg/l) (all from Sigma), which had no effect by themselves on the activity of the antibody.

4.3. RESULTS

A. Thyroidal effects of GH

i. *In vivo*: Growth hormone treatment reduced thyroid weight to 76 ± 8% of control levels ($P < 0.05$, (n=16)) and induced the expected rise in plasma T_3 and decline in plasma T_4 between 60 and 120 min post-injection ($P<0.05$, (n=16)) (data not shown). *In vitro* basal and TSH-induced T_4 release from dissected thyroid glands was also reduced in a dose-related manner (Figure 4. 2). Daily GH injections of 10 µg or 100 µg/d for

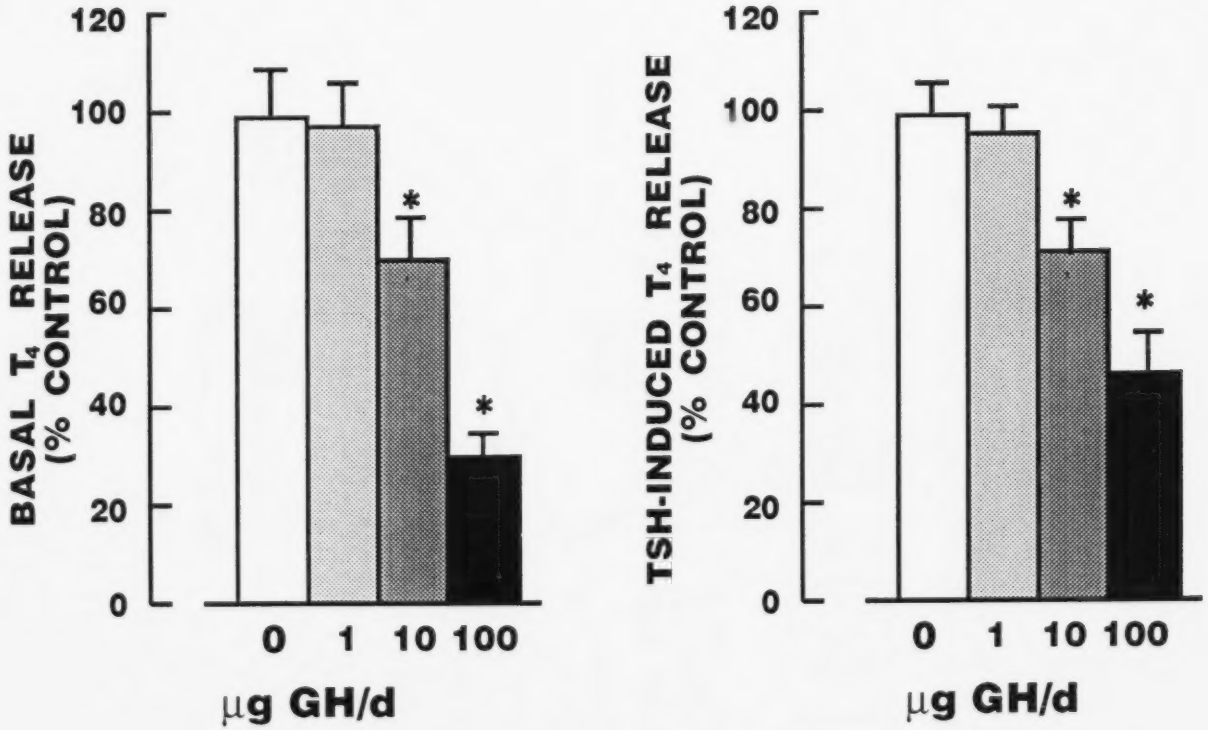

Figure 4.2. Basal and TSH-induced T_4 release from thyroid glands isolated from euthyroid 4 week-old White Leghorn chickens treated with 0, 1, 10 or 100 µg of recombinant chicken GH for 14 days. Thyroid glands were collected 2h after the last GH injection and individually incubated from 3h in Medium 199 in the absence or presence of TSH (200 mU/ml). T_4 concentrations in thyroid-conditioned media were quantified by radioimmunoassay. Values are means +/- S.E.M. (n=16 replicates per group). T_4 release in GH-treated birds was compared to that in untreated birds by ANOVA (*, $p<0.05$).

14 d reduced basal T_4 release to 70% or 30% of control levels (respectively)($P < 0.05$) and reduced TSH-stimulated release to 70% and 45% of control levels (respectively) ($P < 0.05$). Lower concentrations of GH (1 µg/d) had no effect on basal or stimulated T_4 release.

ii. *In vitro:* Incubation of isolated thyroid glands with GH ($1x10^{-9}$ - $1x10^{-5}$ mol/l) did not alter basal T_4 release but significantly ($P < 0.05$) reduced thyroid responsiveness to TSH at higher concentrations (Figure 4.3). TSH-stimulated T_4 release was 260% of basal levels in the absence of GH, but was reduced to 210% and 185% of basal levels in the presence of 10^{-6} mol and 10^{-5} mol/l (respectively).

B. The Thyroid GHR

i. Radioligand Binding: ^{125}I-labelled rcGH bound specifically to membranes of chicken thyroid glands. Total binding was approximately 10% of the total counts and non-specific binding was always <20% of the amount specifically bound. In the presence of increasing amounts of tracer, its specific binding to a constant amount of thyroid membranes was saturable. The specific binding of tracer to thyroid membranes was competitively displaced by unlabelled oGH to a binding site of intermediate-affinity (association constant (Ka) = 2.9×10^{8} mol/l^{-1}) and low-capacity (binding capacity (Bmax) = 5.43×10^{-11} mol/l) (Figure 4.4).

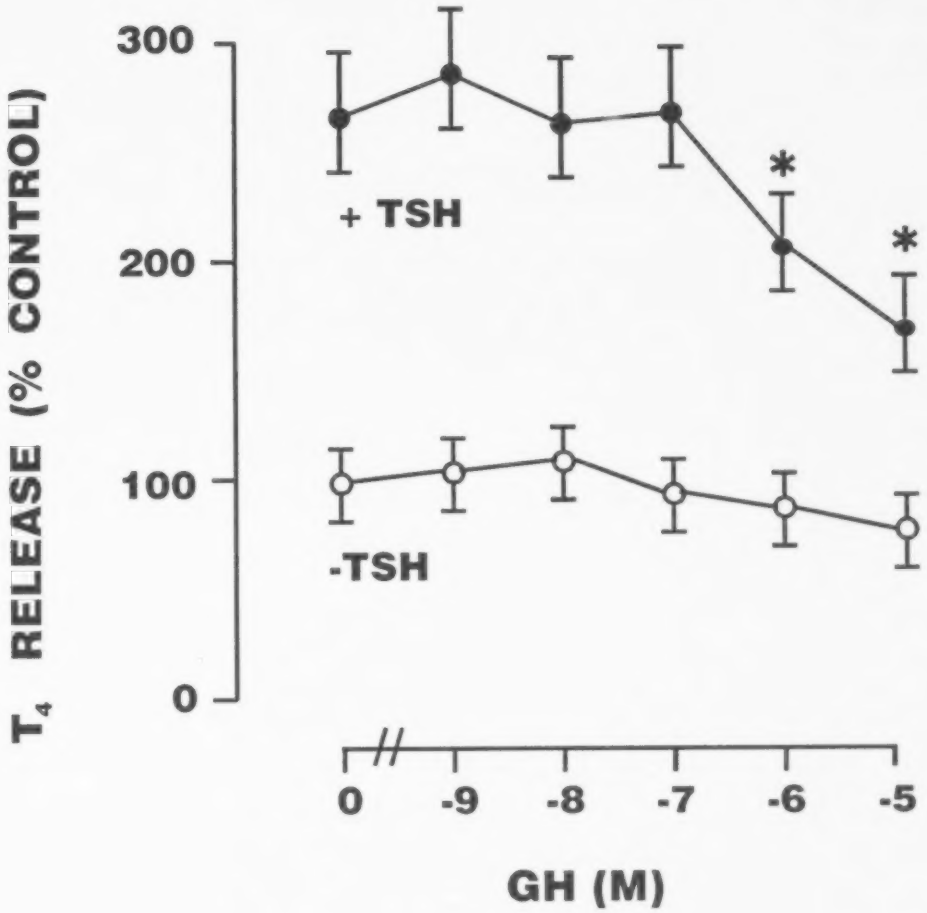

Figure 4.3. T_4 release from isolated thyroid glands incubated in the presence (filled circles) or absence (unfilled circles) of TSH (200 mU/ml) and in the presence of 0 - 10^{-5} M recombinant chicken GH. T_4 concentrations in thyroid-conditioned media were quantified by radioimmunoassay. Values are means +/- S.E.M. (n= 16 replicates per group). T_4 release from GH-treated pituitary glands was compared with that from untreated pituitary glands by ANOVA (*, $p<0.05$).

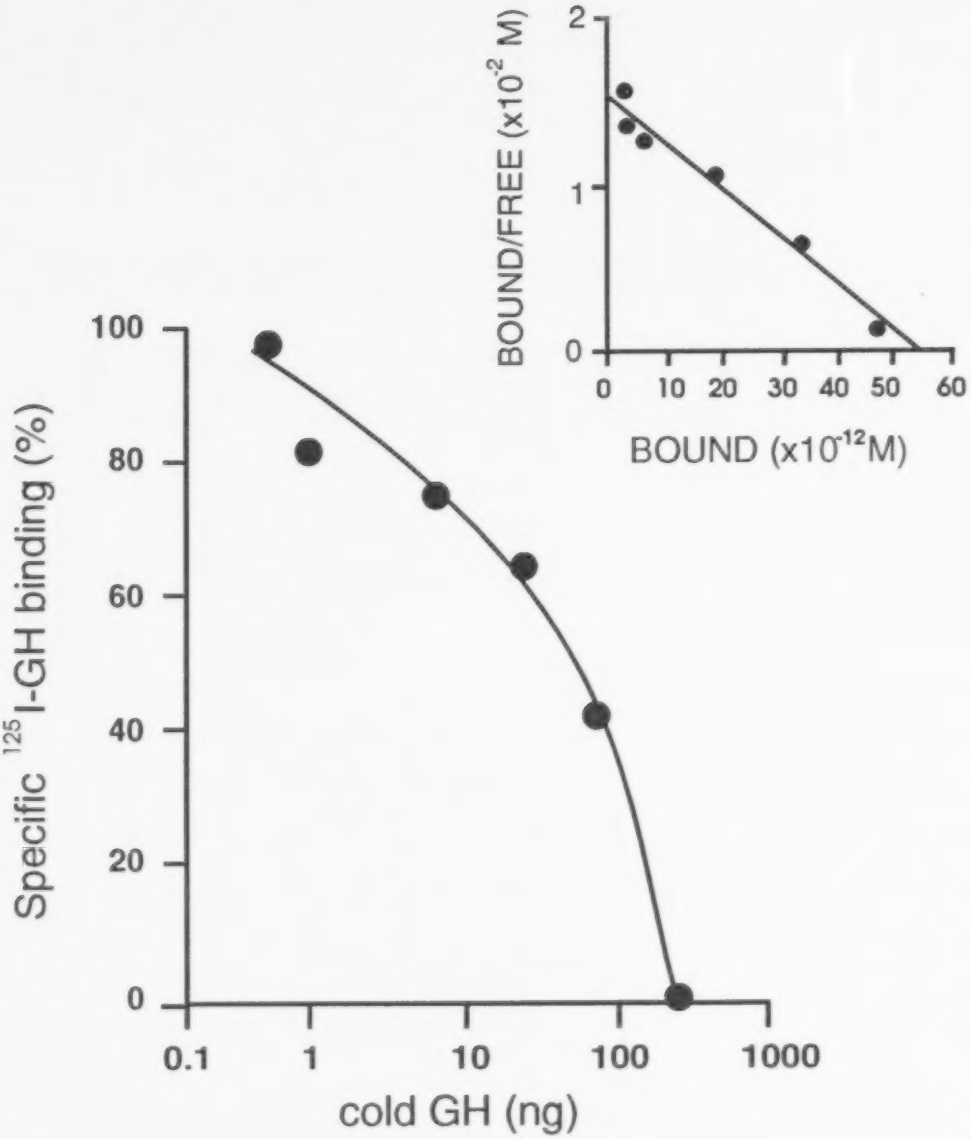

Figure 4.4. Specific binding of ^{125}I-labelled recombinant DNA-derived chicken GH (rcGH) to plasma membranes of the chicken thyroid gland in the presence of ovine GH. Values are means of quadruplicate measurements. The inset is the Scatchard plot of the binding data.

ii. GHR mRNA - Characterization: The cRNA probe specifically hybridized with thyroidal RNA moieties of approximately 4.4 kb, 2.7 kb and 1.0 kb (Figure 4.5a). Similar moieties were also present in chicken liver. In both tissues most of the hybridization (approximately 43%) occurred with the 4.4 kb transcript, however, in overexposed blots the 2.7 kb and 1.0 kb transcripts were responsible for 31% and 26% of total hybridization, respectively. This hybridization was specific since no signal was detected using the sense probe (data not shown).

In the presence of 3′- and 5′-oligonucleotide primers for the extracellular and transmembrane domains of the chicken liver GHR cDNA (Figure 4.1), a moiety of approximately 500 bp was amplified from cDNA synthesized from chicken thyroid extracts (Figure 4.6). When chicken thyroid cDNA was digested with *Nco*I or *Bam*HI, bands of approximately 350 bp and 150 bp or 325 bp and 175 bp (respectively) were observed (Figure 4.6), as would be expected for the digestion of the cGHR cDNA fragment. In a similar manner, a fragment of the expected 800 bp was amplified by primers encoding a portion of the intracellular domain (Figure 4.6). As expected, *Eco*RI or *HaeIII* digestion of the PCR product generated fragments of approximately 530 bp and 275 bp or 469 bp and 337 bp (respectively) (Figure 4.6). The results of both sets of amplifications and digestions mirrored those observed with hepatic cDNA (data not shown). In both PCR reactions, these fragments were not generated when the RNA was not transcribed by Superscript (Figure 4.6) or was absent (data not shown).

iii. GHR mRNA - Regulation: Thiourea treatment resulted in signficantly higher GHR mRNA levels (1.16 ± 0.05 aribitrary units (A.U.)) than in saline-treated birds (0.89 ± 0.12 A.U.) ($p<0.05$(n=4)) as determined by laser densitometry of Northern Blots (Figure 4.5b). This increase in hypothyroid birds was accompanied by a 66.7% augmentation in thyroid size ($p<0.001$ in comparison to controls (n=12)). Conversely, despite a 26.3% decrease in thyroid weight in hyperthyroid birds ($p<0.01$ in comparison to controls (n=12)), GHR mRNA levels were not significantly different from those in control birds (0.89 ± 0.12 A.U. in controls; 0.78 ± 0.005 A.U. in T_3-treated birds (n=4)) (Figure 4.6b). Body weight was unaffected by either treatment.

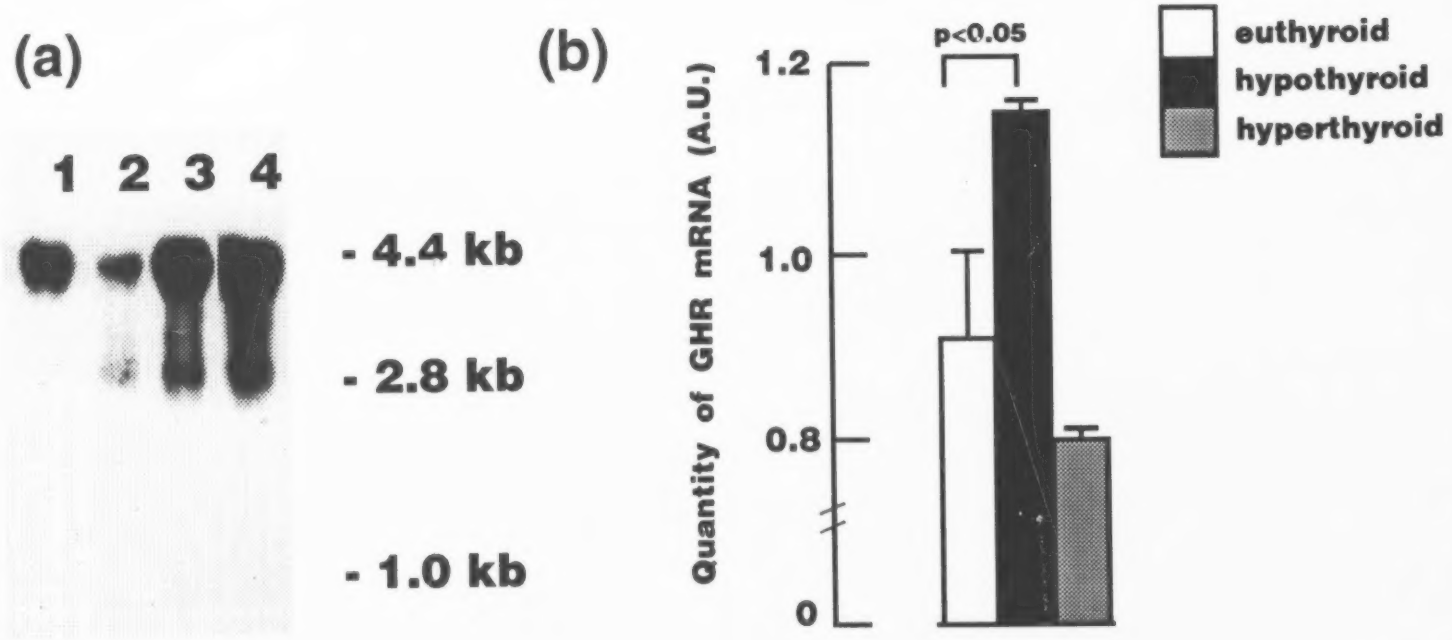

Figure 4.5. (a) A representative Northern Blot demonstrating the cross-hybridization of a rabbit GHR cRNA probe to total RNA extracted from the thyroid glands of euthyroid (lane 1), hyperthyroid (lane 2) and hypothyroid (lane 3) White Leghorn chickens. A 1.0 kb transcript was also observed in the autoradiograph was overexposed. Hybridization to hepatic RNA is shown in lane 4 for comparative purposes. Sizes were determined by comparison with the migration of RNA size markers. (b) The amount of the 4.4 kb GHR mRNA was quantified by laser densitometry and expressed relative to the amount of gamma-actin mRNA (not shown) to correct for slight differences in loading. Values are means +/- S.E.M. of 12 thyroid glands divided into 4 pools.

Figure 4.6. Ethidium bromide-stained 1.4% agarose gel of cDNA reverse-transcribed from total chicken thyroid RNA and amplified by the polymerase chain reaction with 3'- and 5'- oligonucleotide primers (khu 9 and 10) encoding the extracellular domain of the chicken liver GHR cDNA (lane 1). EcoRI (lane 2) and NcoI (lane 3) digests of the PCR products derived from thyroid cDNA samples are also shown. The cDNA was also amplified in the presence of 3' and 5' primers for the intracellular domain (khu 11 and 12) (lane 5) and digested by BamHI (lane 6) and HaeIII (lane 7). When Superscript was omitted from the original reverse transcription mixture, no amplification was observed with either the extracellular (lane 4) or intracellular (lane 8) primers. The size of the various fragments was determined by comparison with the migration of HaeIII digested pUc18 and EcoRI/BamHI-digested lambda size markers.

Iv. GHR - Localization: Immunocytochemical staining of 16 um thyroid sections from euthyroid birds revealed the presence of GHR-positive cells in both follicular epithelial and parafollicular cell populations (Figure 4.7a). The intensity of the signal was more intense in parafollicular than in follicular cells and GHR cells were not present ubiquitously throughout the gland, but were localised in certain follicles. Staining was eliminated if the primary antibody was pre-absorbed by prior incubation with chicken liver membranes (data not shown) or when replaced by non-immune rabbit serum (Figure 4.7b).

4.4. DISCUSSION

Although it is well-established that GH lowers circulating T_4 concentrations at extra-thyroidal sites (Decuypere & Kuhn, 1988; Darras *et al.*, 1992; Darras *et al.*, 1993), the thyroid gland has generally been considered to be resistant to GH action, as GH administration to GH-deficient patients did not affect thyroidal T_4 or T_3 secretion (Jorgensen *et al.*, 1992). Moreover, thyroidal development in transgenic mice was enhanced by the partially lactogenic hGH but not by the somatogenic bGH transgene, suggesting a role for prolactin but not GH in thyroid function (Mayerhofer *et al.*, 1990). This conclusion is not supported by the results of the present study, which clearly demonstrates inhibitory thyroidal actions of GH that are likely to be receptor-mediated. Thus although not previously considered as a GH target-tissue (Waters *et al.*, 1990; Cramer & Talamantes, 1993; Kelly *et al.*, 1993), these results indicate that thyroid glands are sites of GH action. This possibility is strongly supported by the presence of GH-binding sites in thyroidal tissue, in which the GHR gene is transcribed. The up-regulation of GHR/GHBP gene expression in hypothyroid birds is also suggestive of a physiological role for GH in this tissue. The GHR gene in the thyroid gland would appear to be homologous to that in the liver, with extracellular and intracellular domains of sequence identity. The transcription of the GHR gene in the thyroid gland would also appear to be similar to that in other central and peripheral tissues, resulting in the generation of multiple transcripts. The major transcript, as in other tissues, has a molecular size of

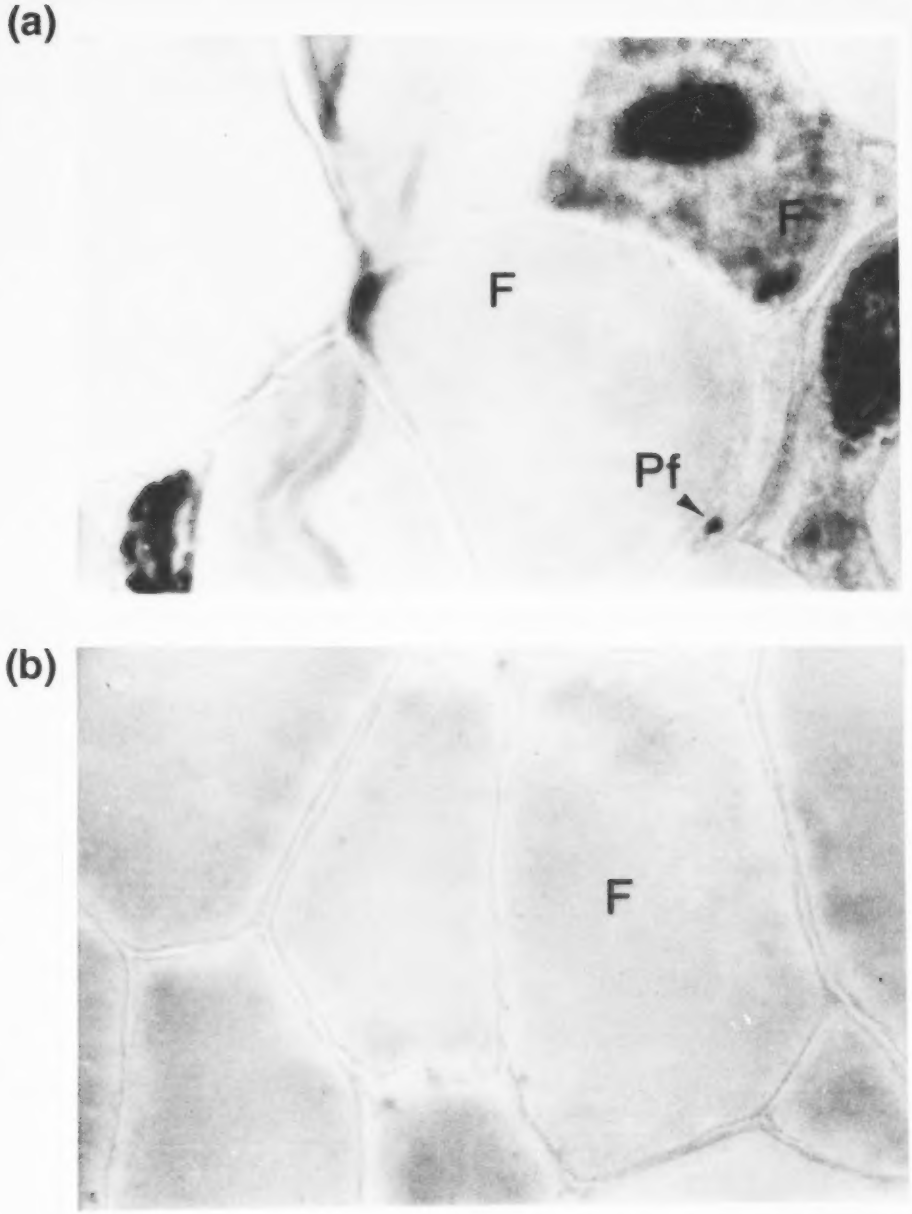

Figure 4.7. Immunocytochemical staining of chicken thyroid glands. (a) 10-16 um frozen sections were cut from the fixed, cryoprotected thyroid glands of euthyroid chickens and stained with a chicken GHBP antibody. Immunoreactive cells are stained dark gray. (b) No staining was observed if the sections were incubated with non-immune rabbit serum or with antibody pre-absorbed with chicken liver membranes (data not shown). Magnification = 1000 x.

approximately 4.4 kb, and probably codes for the full-length receptor (Hull *et al.*, 1993).

Two smaller, less abundant transcripts of 2.7 kb and 1.0 kb were detected. The 2.7 kb transcript may reflect a GH binding protein of smaller molecular size, since signals encoding heterogenous GHR proteins have been described (Tiong & Herington, 1991; Oldham *et al.*, 1993). It may result from alternate polyadenylation in the 3' untranslated region (Burnside *et al.*, 1991), alternate splicing (Bingham *et al.*, 1994) or degradation of the larger product. The 1.0 kb transcript, on the other hand, appears to match that shown to arise from the alternative usage of a poly (A) signal found within the cGHR coding region (Oldham *et al.*, 1993). This transcript may also encode a GH binding protein, although it would be severely truncated (Oldham *et al.*, 1993). Whether either of these putative protein products corresponds to GH binding proteins found in chicken plasma (Vasilatos-Younken *et al.*, 1991) remains to be seen.

The translation of thyroidal mRNA into binding proteins with the extracellular GHR motif was, however, demonstrated immunocytochemically. Immunoreactivity for the GHR/GHBP was widely distributed throughout the thyroid gland and was present in both follicular and parafollicular cells. Immunoreactivity was not, however, detected in all thyroid follicles, although it was present in all the cells of immunoreactive follicles. While differences in GHR/GHBP abundance may account for this heterogeneity, this finding may indicate functionally-distinct subpopulations of thyroid follicles that were hithero considered to be homogeneous. The thyroidal cells may also utilize intracellular GHBPs to competitively modulate GH action (Harvey & Hull, 1994) or to internalize circulating GH, as recently described for adipocytes (Goodman *et al.*, 1993). Thyroidal cells may also secrete GHBPs, which have been shown to be present in the peripheral plasma of these birds (Vasilatos-Younken *et al.*, 1991). However, while extracellular GHBP immunoreactivity was detected in thyroidal colloid, it was apparently not present in the extracellular spaces between thyroid follicles. In addition to modulating the release of T_4, the presence of GHR/GHBP immunoreactivity in parafollicular cells suggests other roles for GH in thyroid function. Since parafollicular cells secrete calcitonin and somatostatin which inhibit GH release in birds (Harvey, 1993, and unpublished observations), modulation of parafollicular function may be of importance in the long-loop

feedback regulation of GH secretion.

The presence of GHR/GHBP immunoreactivity in follicular and parafollicular cells is likely to indicate the presence of GH-binding sites, since radiolabelled GH is competitively bound to thyroidal plasma membranes. Although these sites appear to be of lower affinity and higher capacity than the high-affinity, low-capacity site in the liver (Leung *et al.*, 1987), this may reflect the heterogeneous distribution of the immunoreactive cells and/or a differential distribution of authentic receptors and binding proteins of lower affinity. The binding affinity of these sites is, however, comparable to a lower affinity site identified in rat and rabbit liver (Kelly *et al.*, 1974; Posner *et al.*, 1980).

In summary, these results indicate the functional expression of GH receptors/binding proteins in thyroid tissue, in which GH inhibits T_4 release. The thyroid gland would thus appear to be a novel target site of GH-action.

4.6. REFERENCES

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CHAPTER 5: GROWTH HORMONE RECEPTOR GENE EXPRESSION IN SEX-LINKED DWARF LEGHORN CHICKENS: EVIDENCE AGAINST A GENE DELETION[1]

5.1 INTRODUCTION

Sex-linked dwarfism in poultry is due to a recessive gene on the Z chromosome that phenotypically results in mature body weights 30-40% smaller than those of normal chickens (Guillaume, 1976; Tixier-Boichard, Huybrechts, Kühn *et al.* 1989; Decuypere, Huybrechts, Kühn *et al.* 1991). However, as sex-linked dwarfism is thought to have arisen independently in different strains of domestic fowl (Guillaume, 1976), its aetiology may be due to a variety of gene mutations.

In sex-linked dwarf (SLD) Leghorn chickens, genetically selected for egg laying, dwarfism is likely to be due to tissue resistance to growth hormone (GH) action. Although the pituitary GH gene is normally transcribed and the circulating GH concentrations are normal or supranormal in these birds (Scanes, Marsh, Decuypere & Rudas, 1983; Harvey, Scanes & Marsh, 1984), they are abnormally fat (Guillaume, 1976) and are deficient in plasma tri-iodothyronine (T_3) and insulin-like growth factor 1 (IGF-I) (Huybrechts, Darras, Kühn *et al.* 1987; Huybrechts, Michielsen, Darras *et al.* 1989). Since lipolysis (Scanes, 1991) and the production of IGF-I (Goddard & Boswell, 1991) and T_3 (Kühn, Huybrechts, Darras *et al.* 1990) are GH dependent, the GH-receptor (GHR) gene may be deficient or defective in Leghorn SLD's. This possibility is supported by the inability of exogenous GH to promote growth or restore T_3 or IGF-I production in these birds (Bowen, Huybrechts, Marsh & Scanes, 1987; Huybrechts *et al.* 1987; Leung, Styles, Rosenblum *et al.* 1987b; Kühn *et al.*, 1990) and by the greatly diminished binding of labelled GH to the plasma membranes of SLD tissues (Leung *et al.* 1987b; Vanderpooten,

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[1]A version of this chapter has been published: Hull, K.L., Fraser, R.A., Marsh, J.A. and Harvey, S. J. Endocrinol. 1993; 137: 91-98. I was responsible for all of the data described in this paper. J. Marsh supplied tissue samples and R. Fraser provided technical instruction and advice.

Huybrechts, Decuypere & Kühn, 1991). The GHR gene in broiler SLD's genetically selected for meat production is, moreover, aberrantly transcribed or deficient in coding sequences in comparison with normal growing birds (Burnside, Liou & Cogburn, 1991). The possibility that the GHR gene in Leghorn SLDs may also be anomolous was therefore investigated in the present study.

5.2 MATERIALS AND METHODS

A. Tissues

Peripheral (liver, muscle and adipose) and central (hypothalamic and extra-hypothalamic regions of the brain) tissues known to express the GHR gene (Mendelsohn 1988; Attardo & Harvey, 1990) were rapidly dissected from freshly killed Leghorn SLDs (Cornell University flocks) and from a genetically related normally growing (K) strain. The tissues were immediately frozen in liquid nitrogen and stored at -80°C. For comparative purposes tissues were collected from immature (1 and 8 weeks of age), pubertal (16 weeks of age) and adult (>24 weeks of age) birds, since the binding of labelled GH to the hepatic membranes of normally growing birds increases during growth (Leung *et al.* 1987b; Vanderpooten *et al.* 1991).

B. Northern Blot Analysis

Total cellular RNA was extracted from pooled samples of chicken liver, muscle, fat, brain and hypothalami (five birds per group). The tissues were extracted in guanidinium thiocyanate (5.5 mol/l), containing sodium citrate (25 mmol/l), 0.2% (w/v) ß-mercaptoethanol and 0.5% (w/v) sodium lauryl sarcosine (pH 7.0) and collected after centrifugation (125,000 x g, at 15°C for 24 h) through a bed of caesium trifluoroacetic acid (Pharmacia Fine Chemicals, Uppsala, Sweden, density 2.01) containing EDTA (100 mmol/l), pH 7.0 (Okayama, Kawaichi, Brownstein *et al.*, 1987). The RNA was quantified spectrophotometrically and 5 µg aliquots were subjected to electrophoresis in 1% (w/v) agarose and 3.1% (w/v) formaldehyde gels and transferred to nylon membranes by capillary transfer. A rabbit GHR (rGHR) cRNA probe was constructed from a 638 bp *Bam*H1/*Eco*R1 fragment (donated by Dr. W.I. Wood, Genetech, San Francisco, CA, USA)

of the rabbit hepatic GHR cDNA sequence coding for portions of the extracellular and transmembrane domains (Leung, Spencer, Cachianes *et al.* 1987a). The plasmid pRGEM32 (Promega Corporation, Madison, WI, USA) containing this sequence was linearized by *Bam*H1 digestion and transcribed by T7 polymerase using a riboprobe kit (Promega) in the presence of [α-^{32}P] CTP (800 uCi/mmol; New England Nuclear, Mississauga, Ontario, Canada). This probe specifically hybridizes with GHR RNA in chicken tissues (Fraser, Attardo & Harvey, 1990). Transcription of this plasmid with SP6 polymerase (Promega) generates a sense RNA probe that does not hybridize with chicken mRNA (Fraser *et al.* 1990). The cRNA probe was then hybridized with the immobilized RNA in 50% (w/v) formamide (containing NaCl (0.75 mol/l), PIPES (25 mmoles/l) and EDTA (25 mmol/l), 0.2% (w/v) sodium dodecyl sulphate (SDS), 1x Denhart's Reagent (0.1% (w/v) ficoll, 0.1% (w/v) bovine serum albumin, 0.1% (w/v) polyvinylpyrrolidine), salmon sperm DNA (100 ug/ml) and yeast tRNA (100 ug/ml), pH 6.8) for 12 h at 68°C, following a 3-h, incubation in the absence of the probe. The nylon membranes were then washed twice in each of 2x, 0.5x and 0.05x SSC (1xSSC = sodium citrate (0.015 mol/l) and NaCl (0.15 mol/l), pH 7.2) (containing 0.2% (w/v) SDS) at room temperature, 68°C and 68°C, respectively, prior to exposure to Kodak X-OMAT-AR film (Kodak, Rochester, NY, USA) for 3 d. Membranes were then placed between intensifying screens and exposed to Kodak X-OMAT-AR film (Kodak, Rochester, NY, USA) for 1 day (liver and brain RNA) or 7 d (hypothalamus and adipose RNA). To correct for loading error, blots were stripped of the probe in 1 mmol/l Tris.Cl pH 8.0, 1 mmol/l EDTA and 0.1x (v/v) Denhart's Reagent and hybridized with a mouse γ-actin cRNA probe. The degree of specific hybridization of the GHR cRNA probe to hepatic RNA was quantified by laser densitometry and corrected in relation to hybridization of the γ-actin cRNA probe. The degree of hybridization and the age of the birds was correlated by Spearman's Rank Correlation. The sizes of the RNA moieties hybridizing to the probe were determined by comparison with electrophoretically separable size markers (Boehringer Mannheim, Dorval, Quebec, Canada).

C. Polymerase Chain Reaction (PCR)

Total RNA extracted from the liver, muscle, hypothalamus and brain was also reverse transcribed by Superscript (100 U, BRL, Burlington, Ontario, Canada) in the presence of 100 pmol of oligodeoxythymine primer (Boehringer Mannheim), excess nucleotides (10 mmol each of dCTP, dTTP, dATP and dGTP/l, Boehringer Mannheim) and 5x H-RT buffer (BRL). Reactions were also prepared in the absence of RNA or Superscript, to serve as negative controls. The reactions were diluted with double-distilled water (500:1 vol/vol) and aliquots of liver, muscle and brain cDNA (5% of total volume) were added to a PCR cocktail containing 5'-oligomer chicken (c) GHR sense (5'-CCTCGATTTGGATACCATATTGTGTTAAGC-3') and 3'-oligomer cGHR antisense primers (5'- CTGTTACGGCCAGCCCACACACTCCGAAG -3') (15 pmol of each), deoxynucleotides (1.25 mmoles/l of each), 1x PCR reaction buffer (KCl (50 mmol/l), Tris-Cl (10 mmol /l), pH 8.4, $MgCl_2$ (1.5 mmol/l), and gelatin (20 ug/ml) and *Thermus aquaticus* (*Taq*) DNA polymerase (5 U, BRL). The primers were based on the known sequence of a portion of the cGHR transcript coding for the extracellular domain between nucleotides 303 and 798) (Burnside *et al.* 1991). Reverse-transcribed RNA from liver, muscle, hypothalamus and brain was also amplified in the presence of 5' sense (5'-CTGCGGCCGCAGGACCAGTTCCAAAGATTAA-3') and 3' antisense (5'-AAGCGGCCGCGGCAGTAGTGGTAAGGCTTTC-3') oligonucleotides coding for a portion of the intracellular domain of the receptor between nucleotides 844 and 1667 (Burnside *et al.* 1991). The mixtures were overlayed with mineral oil (v/v), and heat-denatured at 94°C for 2 min before 30 cycles of annealing (at 45°C for 1 min), extension (at 72°C for 3 min) and denaturing (at 94°C for 1 min) in a genetic thermal cycler (Precision Instruments, Chicago, IL, USA). The hepatic PCR products were also digested for 2 h with restriction endonucleases (*Bam*HI, *Nco*I, *Hae*III or *Eco*RI) for known cleavage sites in the regions coding for the extracellular (at nucleotides 445 and 473 for *Bam*HI and nucleotide 449 for *Nco*I) and intracellular (at nucleotide 1330 for *Hae*III and nucleotide 1394 for *Eco*RI) domains of the receptor, respectively (Burnside *et al.* 1991). The PCR and digestion products were electrophoresed in 1.4% (w/v) agarose ethidium bromide-stained minigels and compared with *HaeIII*-digested pUC 18 or *Eco RI/BamHI*-

digested lambda size markers. PCR products were purified directly from the amplification mixture usng the Promega Magicprep Kit (Promega) and partially sequenced (Department of Biochemistry, University of Alberta)

D. Radioligand Binding

The binding of GH to liver plasma membranes from adult SLD and K birds was determined as previously described (Fraser *et al.* 1990). Briefly, hepatic plasma membranes isolated from eight chickens were prepared separately by centrifugation and ultracentrifugation after homogenization in 25 mmol Tris.HCl buffer/l, pH 7.4. The membranes were then incubated at room temperature for 4 h with ^{125}I-labelled ovine GH (60 uCi/ug; NIADDK-oGH-14; Bethesda, MD, U.S.A.) in the presence or absence of unlabelled GH (2 ug/ml) to determine non-specific binding. Bound and free radioactivity were separated by centrifugation.

5.3 RESULTS

A. Radioligand Binding

Specific binding of the GH tracer to hepatic membranes derived from the livers of eight adult SLDs (0.5 ± (SEM) 0.27 % of total counts incubated) was significantly less ($p<0.001$, Student's t-test) than that to membranes derived from eight controls (K strain) (17.6 ± 0.8% of total counts) (data not shown). In both cases the non-specific binding was < 10 % of the total counts.

B. Northern Blot Analysis

The rGHR cRNA probe specifically hybridized with a major transcript of 4.4 kb found in total RNA isolated from the liver of K and SLD birds (Figure 5.1). An age-related increase in the amount of this hybridizing moiety was apparent in 2 separate blots (K: r_s = 0.952 (n=8) $P < 0.01$; SLD: r_s = 0.976 (n=7) $P<0.01$, Spearman's Rank Correlation) (Figure 5.2). A smaller transcript of 2.7 kb hybridizing with the rGHR cRNA probe was also evident in hepatic RNA from both strains when the same blots were exposed for 72 h (data not shown). In each strain, this transcript accounted for

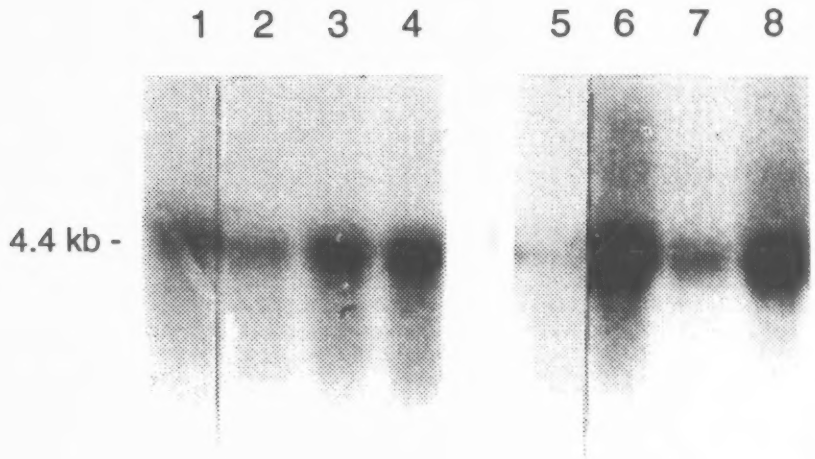

Figure 5.1. Cross-hybridization of a rabbit cRNA probe to hepatic total RNA extracts from Cornell sex-linked dwarf (SLD) chickens of 1 week (lane 1), 4 weeks (lane 2), 16 weeks (lane 3) of age and adult chickens (lane 4). Lanes 5-8 contain total hepatic RNA extracts from Cornell K-line chickens aged 1 week (lane 5), 4 weeks (lane 6), 16 weeks (lane 7) and adults (lane 8). The blot was exposed to Kodak X-OMAT AR film for 24 h. Sizes were determined by comparison with the migration of RNA size markers.

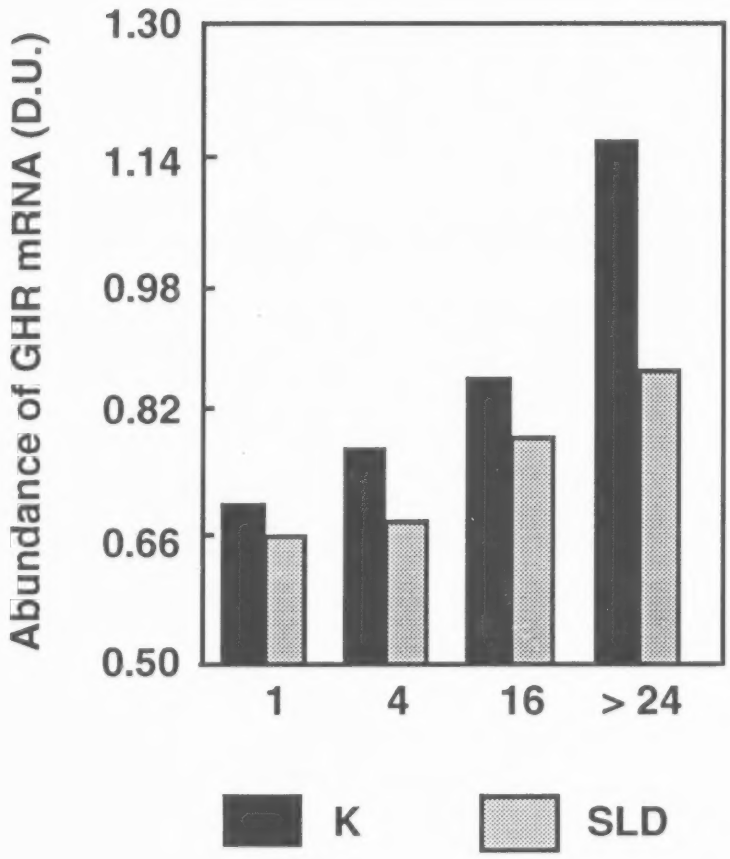

Figure 5.2. GH receptor (GHR) mRNA (4.4 kb) abundance in 1-, 8-, 16- and 24-week-old Cornell (K: solid bars) and sex-linked dwarf (SLD: cross-hatched bars) chickens. Hybridization was quantified by laser densitometry and normalized with respect to hybridization of a mouse gamma-actin probe.

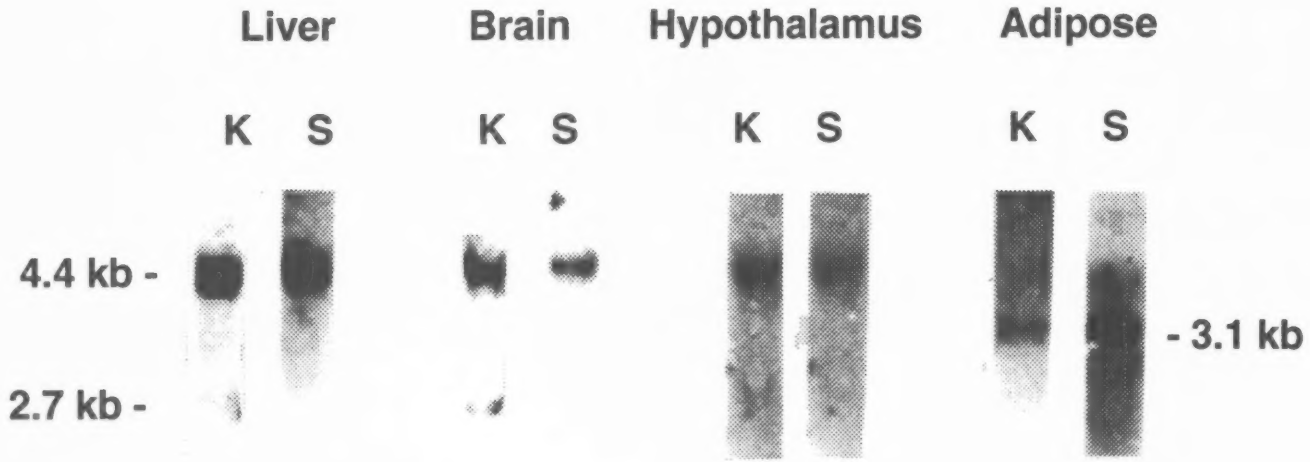

Figure 5.3. Cross-hybridization of a rabbit cRNA probe to total RNA extracted from the liver, brain, hypothalamus and adipose of control Cornell (K) and sex-linked dwarf (S) Leghorn chickens. Sizes were determined by comparison with the migration of RNA size markers.

approximately 45% of the total hybridization. The larger transcript was ubiquitously present in other tissues of both strains, although the smaller transcript was only detected in the brain of the K strain and was undetectable in hypothalamic and fat RNA from SLD and K birds when the blots were exposed for 7 days (Figure 5.3). Hybridization with an intermediate transcript of 3.1 kb was also evident in RNA extracted from the adipose tissues of both strains.

C. Polymerase Chain Reaction

A single moiety of 804 bp was generated when cDNA reverse-transcribed from hepatic, muscle and brain RNA from SLD and K birds was amplified by PCR in the presence of 3` and 5` oligonucleotide primers coding for the intracellular domain of the GHR (Figure 5.4). Digestion of both hepatic PCR products with *EcoRI* (Figure 5.4) and *Hae*III (data not shown) produced fragments of the expected sizes (533 bp and 271 bp, 469 bp and 337 bp, respectively). In the presence of 3` and 5` primers coding for portions of the extracellular and transmembrane regions of the GHR, a single moiety of the appropriate size (496 bp) was amplified from hepatic, muscle, brain and hypothalamic cDNA (Figure 5.5). Digestion of both hepatic PCR products with *BamHI* generated identical fragments (325 bp and 171 bp) of predicted sizes (Figure 5.5). Digestion of the cDNA with *Nco*I also produced fragments of the expected sizes (350 bp and 150 bp) (data not shown). In both PCR reactions, these fragments were not generated when the RNA was not transcribed by *Superscript* or was absent (results not shown). The sequence of the extracellular PCR fragment between nucleotides 393 and 621 and the intracellular PCR fragment between nucleotides 903 and 1232 was more than 99% identical in K and SLD samples, corresponding to the published sequence (Burnside *et al.* 1991) (Figure 5.6).

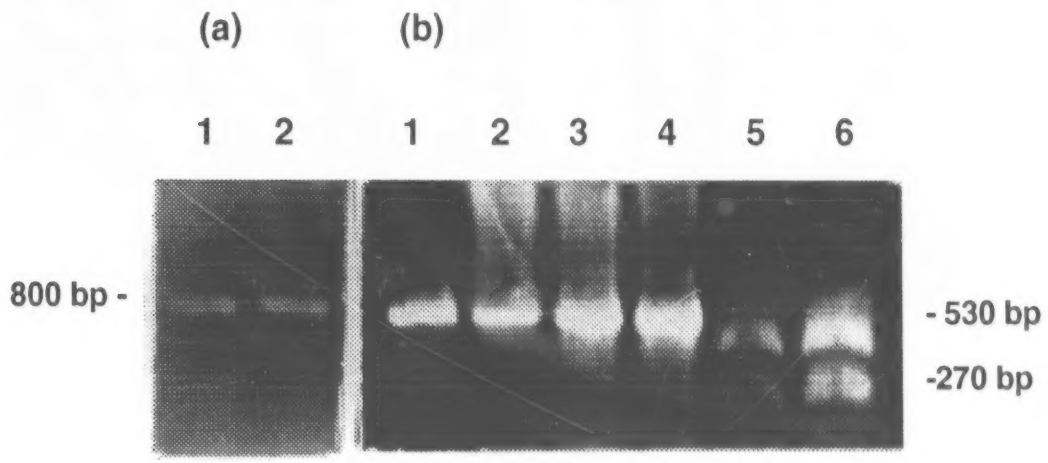

Figure 5.4. (a) Ethidium bromide-stained 1.4% agarose gel of cDNA reverse-transcribed from control Cornell (K) (lane 1) and sex-linked dwarf (SLD) Leghorn chickens (lane 2). Brain cDNA was amplified by the polymerase chain reaction (PCR) with 3' and 5' oligonucleotide primers for the intracellular domain of the chicken liver GH receptor cDNA prior to electrophoresis. (b) Total RNA extracted from K (lane 1) and SLD (lane 2) muscle and K (lane 3) and SLD (lane 4) liver was similarly reverse-transcribed and amplified. EcoRI digests of the PCR products derived from adult K (lane 5) and SLD (lane 6) hepatic cDNA samples are also shown. The size of the various fragments was determined by comparison with the migration of HaeIII-digested pUC18 and EcoRI/BamHI-digested lambda size markers.

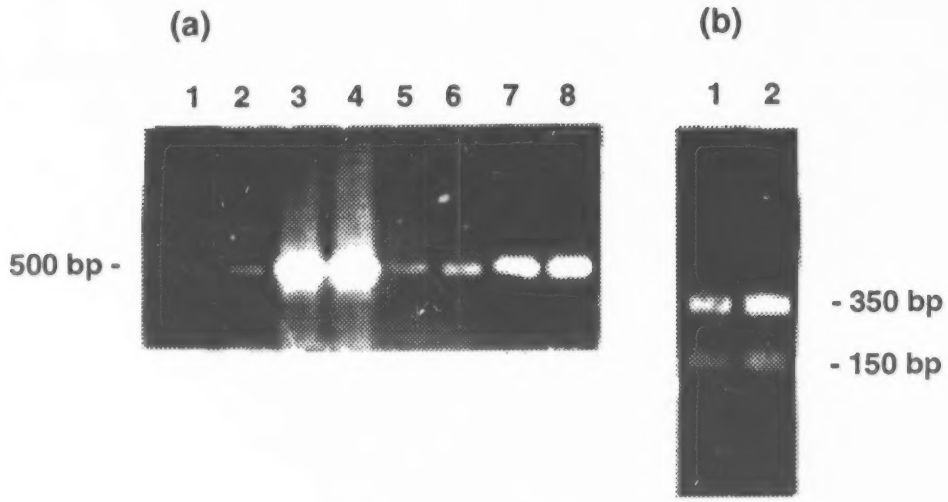

Figure 5.5. (a) Ethidium bromide-stained 1.4% agarose gel of cDNA reverse-transcribed from total RNA extracted from control (K) (lane 1) and sex-linked dwarf (SLD)(lane 2) hypothalami, K(lane 3) and SLD (lane 4) liver, K (lane 5) and SLD (lane 6) brain, and K (lane 7) and SLD (lane 8) muscle. The cDNA was amplified by the polymerase chain reaction with 3' and 5' oligonucleotide primers for the extracellular and transmembrane domains of chicken liver GH receptor cDNA. (b) BamHI digests of K (lane 1) and SLD (lane 2) amplified hepatic cDNA. The sizes of the various fragments were determined by comparison with the migration of HAEIII-digested pUC18 and EcoRI/BamHI digested lambda size markers.

(A)

903
K:GAAAGCTAGATGAAGTGAACTCCATCTTAGCCAGCCATGACAACTACAAGACACAGC
S: GAAAGCTAGATGAAGTGAACTCCATCTTAGCCAGCCATGACAACTACAAGACACAGC

961
K:TATACAATGATGACTTGTGGGTTGAGTTTATTGAATTGGACATAGATGACTCCGATG
S: TATACAATGATGACTTGTGGGTTGAGTTTATTGAATTGGACATAGATGACTCCGATG

1018
K:AAAAGAACAGAGTCTCAGATACTGACAGGCTCCTGAGTGACGATCATCTGAAGTCAC
S: AAAAGAACAGAGTCTCAGATACTGACAGGCTCCTGAGTGACGATCATCTGAAGTCAC

1075
K:ACAGTTGCTTGGGAGCCAAGGATGATGATTCTGGACGTGCCAGTTGTTATGAACCAG
S: ACAGTTGCTTGGGAGCCAAGGATGATGATTCTGGACGTGCCAGTTGTTATGAACCAG

1132
K:ATATTCCAGAGACAGACTTCAGTGCAAGCGACACATGTGATGCCATCTCTGATATTG
S: ATNTTCCAGAGACAGACTTCAGTGCAAGCGACACATGTGATGCCATCTCTGATNTTG

1199
K:ATCAGTTCAAGAAGGTAACTGAAAAAGAAGAGGNTCTCTTGTG
S: ATCAGTTCAAGAAGGTAACTGAAAANGAAGAGGATCTCTTGTC

(B)

393
K:CCTGATCCCCCTGTGCACCTTAACTGGACTCTGCTAAATACTAGTCAAACTGGGATCC
S: CCTGATCCCCCTGTGCACCTTAACTGGACTCTGCTAAATACTAGTCAAACTGGGATCC

451
K:ATGGGGATATTCAAGTACGATGGGATCCACCACCAACAGCAGATGTTCAGAAAGGA
S: ATGGGGATATTCAAGTACGATGGGATCCACCACCAACAGCAGATGTTCAGAAAGGA

507
K:TGGATTACTCTGGAGTATGAATTGCAGTACAAAGAAGTTAATGAGACAAAATGGAA
S: TGGATTACTCTGGAGTATGAATNGCAGTACAAAGAAGTTAATGAGACAAAATGGAA

563
K:GGAGTTAGAACCCAGGTTCTCAACAGTGGTTCCACTGTTTTCTCTGAAGATGGGAAG
S: GGAGTTAGAACCCAGGCTCTCAACAGTGGTTCCACTGTTTTCTCTGAAGATGGGAAG

619
K:AG
S: AG

Figure 5.6. Partial sequences of the polymerase chain reaction products amplified from cDNA reverse transcribed from RNA isolated from control Cornell (K) and sex-linked dwarf (S) birds. Sequences coding for the (a) intracellular and (b) extracellular domains of the receptor were amplified using primers khu11 and khu12 or khu9 and khu10, respectively. N represents sites in which the identity of the nucleotide was unclear.

5.4 DISCUSSION

These results clearly demonstrate that the GHR dysfunction in Leghorn SLDs is not due to a GHR gene deletion. The GHR gene transcripts in SLD tissues were of identical size to those in the K strain, as were the PCR products generated with oligonucleotide primers for the extracellular and intracellular sequences of the GHR. The coding sequences for portions of the GHR cDNA in Leghorn SLDs must therefore be very similar to those in the K strain, and cleavage sites for the restriction endonucleases *Bam*HI, *Nco*I, *Hae*III and *Eco*RI must be similarly located on the gene. These results are, however, in contrast with those of Burnside *et al.* (1991), who found a 1.0-1.7 kb deletion in the GHR transcript of SLD broilers by Northern Blot analysis and a GHR cDNA polymorphism by Southern blot analysis. It is, however, possible that undetected base pair point mutations occur along the length of the GHR in SLD Leghorn chickens, small deletions occur in areas outside those covered by the oligonucleotide primers or the untranslated regions of the gene are structurally defective. A dysfunction or deficiency in the translation of the GHR message may also account for the failure of labelled GH to bind to tissue membranes from Leghorn SLD's and the inability of exogenous GH to stimulate IGF-I or T_3 production in these birds.

The etiology of dwarfism in Leghorn and broiler SLDs is therefore likely to be due to different lesions in the GHR gene. Sex-linked dwarfism in poultry would thus appear to resemble closely Laron dwarfism in humans, in which subpopulations with or without discontinuous deletions or point mutations of the GHR gene have been identified (Amselem, Duquesnoy, Attree *et al.* 1989; Godowski, Leung, Meacham *et al.* 1989). The SLD phenotype in poultry may thus result from a plurality of GHR gene anomalies, and provide a valuable model for the aetiology and treatment of this human disease. Elucidation of the different lesions of the GHR gene in SLD poultry may also provide an insight into the signal transduction pathways that mediate GH action.

As in normal growing birds, production of the hepatic GHR transcript in SLDs increases with age. In contrast, the age-related increase in GH-binding to hepatic tissue (Leung *et al.* 1987b; Vasilatos-Younken, Gray, Bacon *et al.* 1990; Vanderpooten *et al.*

1991) is not observed in Leghorn SLDs. This difference may result from the age-related decline in circulating GH concentrations in normal birds, since this is attenuated in Leghorn SLDs (Scanes *et al.* 1983; Huybrechts *et al.* 1989; Tixier-Boichard, Huybrechts, Decuypere *et al.* 1992). This decline in GH secretion may allow an up-regulation of the hepatic GHR, which is known to be down-regulated by exogenous and endogenous GH (Leung, Taylor, Wein & Van Idersteine 1986; Vasilatos-Younken *et al.* 1990; Vanderpooten, Darras, Huybrechts *et al.*1991). The maturational decline in GH secretion in normal birds is thought to be due to the acquisition of inhibitory hypothalamic control (Harvey, Fraser & Lea, 1991) and possibly autoregulation mediated through hypothalamic GH receptors (Attardo & Harvey, 1990; Fraser *et al.* 1990). Although Leghorn SLDs have hypothalamic GHR transcripts (Figure 5.2), GHRs in this tissue may be defective, in view of the impaired binding of GH to Leghorn SLD livers.

In addition to the major GHR transcript, a smaller 2.7 kb transcript was also observed in liver and brain mRNA from normal growing Leghorns and in SLD hepatic mRNA. This moiety may result from the tissue degradation of the 4.4 kb transcript, or indicate alternative transcription of the GHR gene in a tissue and/or strain-specific way. The heterogeneity of the GHR is well-established; GH receptors having a variety of affinities (Hughes, 1979), molecular weights (Wallis, Daniels & Webb, 1987) and subcellular locations (Herington, Ymer, Roupas & Stevenson, 1988) have been identified. Serum binding proteins of high (Fraser *et al.* 1990; Jones, Anderson & Vasilatas, 1990) and low affinity (Leung *et al.* 1987a; Baumann, Ambern & Shaw, 1988; Spencer, Hammond, Henzel *et al.* 1988; Huybrechts *et al.* 1989; Smith, Kuniyoshi & Talamantes, 1989; Baumann, 1991) also exist with varying degrees of homology to the membrane-bound hepatic receptor. Multiple mRNAs coding for the GHR/GH-binding protein have been isolated in rat liver (Donner, 1983; Tiong, Freed & Herington, 1989) and mouse liver (Smith *et al.* 1989). In brain and liver samples, the various GHR transcripts are present in similar ratios in SLD and K birds. In both strains only the large GHR transcript was detected in the hypothalamus and the 3.1 kb GHR transcript was confined to adipose mRNA. Transcription of the GHR gene or the intracellular processing of the transcripts may therefore be quantitatively or qualitatively tissue-specific.

In conclusion, sex-linked dwarfism in poultry is due to a plurality of causes. In contrast to broiler SLDs, transcription of the GHR gene of SLD Leghorn chickens is not anomalous; regions of the gene coding for the extracellular and intracellular regions of the receptor are present and largely intact.

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CHAPTER 6: GROWTH HORMONE (GH)-BINDING PROTEINS IN GH-RESISTANT GUINEA PIGS[1]

6.1. INTRODUCTION

Although guinea pigs are not GH-deficient and are able to secrete GH in a pulsatile fashion, they grow normally in the absence of pituitary growth hormone (GH) (Mitchell *et al.*, 1954; Daughaday, 1992). The guinea pig has thus been considered to be GH-resistant, especially as extracts of guinea pig pituitary glands and preparations of bovine or monkey GH are incapable of stimulating bone growth, body weight or ornithine carboxylase synthesis in this animal (Mitchell *et al.*, 1954; Daughaday *et al.*, 1986), yet they are effective growth promoters in hypophysectomized rats (Knobil & Greep, 1959). This resistance to GH action is likely to be due to a GH receptor (GHR) or post-receptor defect. The GHR gene is, however, expressed in guinea pigs, in which membrane binding sites for radiolabelled GH are abundantly present (Harvey & Fraser, 1992). A defect in GHR processing or in GHR transcription may, nevertheless, account for the GH resistance of guinea pigs, since they appear to lack the circulating GH binding proteins (GHBPs) that are derived from separate GHR gene transcripts (Baumbach *et al.*, 1989; Cramer & Talamantes, 1993) or produced by the proteolytic cleavage of membrane GHR receptors (Trivedi & Daughaday, 1988; Cramer & Talamantes, 1993). A GHBP deficiency may thus be responsible for the impaired action of GH in guinea pigs.

Although the importance of pituitary GH in inducing growth is well-established, the role of the GHBP is poorly understood. *In vivo,* the biological actions of GH appear to be enhanced in the presence of circulating GHBPs (Harvey & Hull, 1994). For example, slow growth velocity is associated with low or absent serum GHBP's in many populations of Laron-type dwarfs (Fuh *et al.*, 1992; Amselem *et al.*, 1989) and pygmies (Baumann *et al.*, 1989) and in some patients with idiopathic short stature (Carlsson *et al.*,

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1992; Fontoura *et al.*, 1992). In contrast, the efficacy of exogenous GH in normal, hypophysectomized and GH-deficient rats is enhanced after *in vitro* complexing with GHBP or after the *in vivo* infusion of exogenous GHBP (Robinson *et al.*, 1993). The therapeutic effects of GH in GH-deficient children are similarly augmented by GHBP supplementation (Martha *et al.*, 1992). An absence of circulating GHBP may thus partly account for the paradoxical GH resistance in guinea pigs (Harvey & Fraser, 1992). This possibility has therefore been investigated in the present study.

6.2. MATERIALS AND METHODS

A. Northern Analysis

The possibility that guinea pigs, unlike rats and mice (Baumbach *et al.*, 1989; Smith *et al.*, 1989), lack a separate GHR transcript that codes for a truncated binding protein was examined by Northern analysis. Polyadenylated RNA was extracted from the frozen (-80°C) livers of adult male guinea pigs (University of Alberta Health Sciences Laboratory Animal Services (HSLAS)) as previously described (Oldham *et al.* 1993). Briefly, total cellular RNA was prepared by acid-phenol extraction and poly(A) RNA was selected by chromatography on oligo (dT) cellulose (Collaborative Genetics, Cambridge, MA). Poly(A) RNA (3 μg) was electrophoresed in 0.8% agarose-formaldehyde gels and transferred to a nylon membrane (Nytran, Schleicher and Schuell, Keen, NH). A cDNA fragment encoding the majority of the extracellular domain of the rat GHR (pRat1-20) was gel-purified and labelled with ^{32}P-dCTP (New England Nuclear, Boston, MA) by random priming. Membranes were prehybridized and hybridized in Church buffer (0.25 M Na_2HPO_4 (pH 7.2), 7% sodium dodecyl sulfate (SDS), 1 mM EDTA and 0.5% BSA), washed in 0.2 X SSC (1 x CCS = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and exposed to autoradiographic film at -80°C in the presence of an intensifying screen. For comparative purposes, poly(A) RNA from adult male Sprague Dawley rats was similarly extracted and analysed under identical conditions.

B. Radioimmunoassay

The possibility that guinea pigs may be deficient in immunoreactive serum GHBPs

was assessed by radioimmunoassay (RIA). A heterologous system was first developed using MAb 263 (Agen Biomedical Ltd., Brisbane, Australia)(raised against the extracellular domain of the rat GHR) as the primary antibody and human GHBP (hGHBP, kindly donated by Dr. J.A. Wells, Genetech, San Francisco) as the standard and chloramine T-radioiodinated tracer. The tracer had a specific activity of approximately 40 μCi/μg and was > 90.0% precipitable with excess primary antibody, which specifically bound approximately 40% of the tracer when present at a final dilution of 1:3000. In contrast, this tracer was not specifically bound by other MAbs (MAb 1, MAb 5, MAb 7; Agen: MAb 16 and MAb 4.3; American Cyanamid, Princeton, NJ) raised against extracellular domains of the GHR nor by antisera raised against the mouse GHBP (kindly donated by Dr. F. Talamantes, Unversity of California at Santa Cruz) nor chicken GHBP (American Cyanamid). The non-specific binding of this tracer varied between 6.1 and 9.4% of the total radioactivity (12 000 - 15 000 cpm) added to the assay tubes, which were incubated for 24h at 4°C prior to the addition (at a final concentration of 1:120) of the goat anti-mouse secondary antibody (Sigma Chemicals, St. Louis, MO). After a further 24h incubation at 4°C, bound and free radioactivity were separated by centrifugation and aspiration of the supernatants. The precipitation of bound radioactivity was assisted by the addition of 100 μl protein A (Tachisorb, Calbiochem Corporation, La Jolla, CA), at a final concentration of 1:120. The hGHBP standard (over the range 2500 - 2.4 ug/l) and serum samples were diluted in 0.05 sodium phosphate buffer (containing 1% bovine serum albumin), pH 7.0, in a sample volume of 200 μl.

GHBP immunoreactivity in guinea pig serum was also assessed in a heterologous RIA in which a polyclonal antibody (p(Ab)1), raised in rabbits against recombinant rat GHBP (Sadeghi *et al.*, 1990), was used as the primary antibody and hGHBP was used as the standard and ^{125}I-labelled tracer. At a final concentration of 1:5000 the specific binding of ^{125}I-hGHBP to this antibody was 26.7 %, whereas non-specific binding was <3%. The same antibody was similarly used in a homologous RIAs in which specific binding of radioiodinated rat GHBP (American Cyanamid) was 34.0% for its binding to p(Ab)1 (at a final concentration of 1:4800) and 17.5% for its binding to MAb 4.3 (at a final dilution of 1:6000). In contrast, the specific binding of the rGHBP tracer to other

monoclonal antibodies against rat GHBP (MAb 1, MAb 5 and MAb 7; Agen Biomedicals and MAb 16, American Cyanamid) was <5%, even at final concentrations of 1:500. This tracer was, however, specifically bound (23.9%) by MAb 263 (at a final dilution of 1:3000).

GHBP immunoreactivity was determined in guinea pig sera obtained locally (HSLAS) and from commercial sources (Sigma; Calbiochem, San Diego, CA; ICN Biomedicals, Costa Messa, CA; Miles Laboratories; Gibco, Grand Island, NY). For comparison, GHBP immunoreactivity was also determined in out-dated human serum and serum from adult pigs, dogs, horses, sheep, goats, cows, rabbits, rats and mice. Serum samples from domestic fowl, turtles (*Chrysemis picta*), toads (*Xenopus laevis*) and fish (flounder, hagfish, ratfish and dogfish) were similarly assayed for immunoreactive GHBP content. GHBP immunoreactivity in guinea pig and rat tissues was also determined in homolgous RIAs for rat GHBP and expressed relative to the protein content of the PBS extracts, determined by the Bradford method (Bradford, 1976).

C. Radioligand Binding

The ability of guinea pig serum to bind GH was determined by radioligand binding studies in which ovine GH (oGH - 14, 1.4 IU/mg; NIDDK, Bethesda, MD) or human GH (NIDDK-hGH-1-1, AFP-4793B, Bethesda, MD) were used as the ^{125}I-labelled tracers. Both GH preparations were radioiodonated by the Chloramine T method to a specific activity of 40-50 μCi/μg and were used within two or three weeks of iodination. The tracers (approximately 50 000 cpm/tube) were incubated in aliquots (50-150 ul) of guinea pig serum at 4°C for 16h or at 22°C for 1-4 h in the presence or absence of unlabelled hormone (10 μg/ml), to determine non-specific binding. The separation of bound and free radioactivity was achieved by immunoprecipitation (Fraser & Harvey, 1992; Barnard & Waters, 1985) or by adsorption with 3% charcoal (Tobar-Dupres *et al.*, 1993). Excess MAb 263 (Agen, at 1:20), p(Ab)1 (Sadeghi *et al.*, 1990)(at 1:400) or MAb 4.3 (Sadeghi *et al.*, 1990)(at 1:400) were used for immunoprecipitation. Radioligand binding sites in guinea pig serum were also assessed by gel filtration on Sephadex G100 (Baumann *et al.*, 1986; Hull *et al.*, 1992) following the incubation of a 1 ml serum pool with

approximately 1 000 000 cpm hGH tracer, in the presence and absence of excess (10 ug/ml) unlabelled hGH.

For comparative purposes, radioligand binding studies were conducted with serum samples from rats, rabbits, dogs and horses.

D. Gel Electrophoresis and Western Analysis

The possibility that guinea pig serum may be deficient in immunoreactive GHBP or contain a GHBP of anomolous size was evaluated by Western Analysis. Aliquots of 1-2 µl of serum or 100 µg of liver tissue (homogenized in 1% w/v sodium dodecyl sulfate (SDS) containing 1 mM PMSF (Sigma) and 10 µg/ml aprotinin (Sigma)) was diluted in gel loading buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue) and subjected to electrophoresis in 15% acrylamide -SDS gels. Guinea pig serum was also depleted of immunoglobulins by a 2h incubation with Tachisorb (Calbiochem) followed by centrifugation at 11 000 g for 1 min. Separated proteins were subsequently electroblotted onto Immobilon P membranes (Millipore, Mississauga, Ont.) in 10 mM 3-[cyclohexylamino]-1-propane-sulfonic acid buffer (Sigma), pH 10, containing 10% methanol. The filter was incubated overnight with the primary antibody in tris-buffered saline (TBS) (10 mM Tris (pH 7.5), 150 mM NaCl) containing 5% non-fat dried milk (polyclonal antibodies, 1:400 - 1:2000; MAb 263 (Agen) and MAb 4.3 (Sadeghi *et al.*, 1990), 1:500-1:1000) following a 1h blocking step in the same buffer. The polyclonal antibody was raised against recombinant rat GHBP (rGHBP) (Sadeghi *et al.*, 1990) and MAb 263 recognizes a common epitope in mammalian GHBPs. MAb 4.3 was raised against a synthetic peptide corresponding to the alternatively spliced, hydrophilic tail of the rat GHBP. Replicate blots were incubated in the absence of primary antibody or with an unrelated monoclonal antibody (raised against *Brucella*; Agen). As a further negative control, some replicate blots were also incubated with antibody that had been pre-absorbed by a 1 h incubation with recombinant rat GHBP (1 - 100 µg/10 ml) (polyclonal antibodies, MAb 263) or with a peptide corresponding to the unique rat hydrophilic GHBP tail (10-100 ug/10 ml) (MAb 4.3). The blot was subsequently washed in TBS (4 x 30 min) and incubated with horseradish

peroxidase-labelled secondary antibody (goat anti-rabbit IgG for the polyclonal antibodies, rabbit anti-mouse IgG for the monoclonal antibodies)(Amersham, Lancashire, England) for 1 h at a dilution of 1:1500. Following four 30 min washes in TBS containing 1% bovine serum albumin (BSA, Sigma), the filter was developed using enhanced chemiluminescence (ECL) (Amersham) and autoradiographed. Molecular mass of the visualized bands was determined by comparison with Rainbow markers (Amersham) for proteins of 14.3 kDa -200 kDa. In some cases the autoradiographic signal was quantified by laser densitometry.

E. Immunoprecipitation

To increase the specificity of GHBP detection, liver or serum samples were also immunoprecipitated prior to Western analysis. 1-100 µl aliquots of serum or liver homogenates were diluted to 1 ml in TBS containing 0.1% Triton X-100 and incubated with 10 µl of p(Ab)2 or MAb 4.3. After an overnight incubation, protein-antibody complexes were subsequently immunoprecipitated with 100 µl protein A (Pansorbin; Calbiochem). The precipitates were then washed twice in wash buffer (10 mM Tris (pH 8.0), 0.14 M NaCl, 0.025% sodium azide) and once in distilled water prior to elution and denaturation of antibodies and GHBP-like immunoreactive proteins with 50 µl gel loading buffer.

6.3. RESULTS

A. Northern Analysis

As expected, Northern analysis of poly(A) RNA extracted from guinea pig and rat liver revealed the presence of a 4.75 kb transcript that specifically hybridized with a rat GHR cDNA probe (Figure 6.1). This corresponds in size to the full-length rat GHR mRNA. Hybridization was also observed with a smaller moiety of 1.2 kb in rat mRNA, corresponding in size to the well-characterised rat GHBP transcript. Quantification of the relative amount of hybridization to the two GHR mRNA variants revealed a GHR:GHBP ratio of 1:1.7. In contrast, a slightly larger, 1.9 kb transcript was detected in guinea pig mRNA which was equally abundant as the 4.4 kb transcript (Figure 6.1).

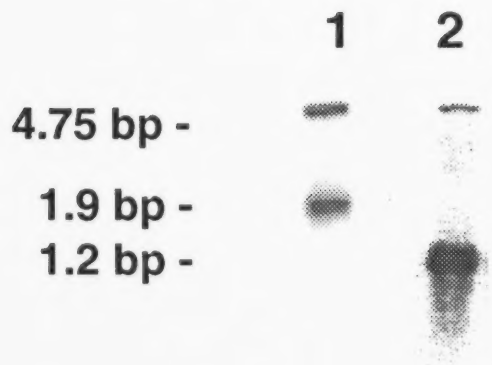

Figure 6.1. Northern analysis of polyadenylated RNA extracted from guinea pig (lane 1) and rat (lane 2) liver. The membrane was incubated with a rat GHR cDNA probe, and the sizes of hybridizing moieties were determined by comparison with RNA size markers.

B. Radioimmunoassay

In a heterologous radioimmunoassay, serial dilutions (1:4 - 1:64) of a guinea pig serum (50 µl - 3.13 µl) were able to completely displace I^{125}-labelled hGHBP binding to MAb 263 in a manner parallel to that of unlabelled hGHBP (Figure 6.2a). Undiluted guinea pig serum or serum diluted 1:1 or 1:2 completely inhibited tracer binding. This displacement of tracer binding was not due to competitive binding to guinea pig IgG or to the non-specific cross-reactivity of guinea pig IgG, since exogenous guinea pig IgG did not displace tracer binding, even at concentrations of 10 mg/ml (data not shown). In this system, the amount of immunoreactive GHBP detected in guinea pig serum was far greater ($P<0.001$) than that detected in rat circulation or in the serum of other mammalian and non-mammalian species (Table 6.1).

Similar results were also obtained in other RIA systems using hGHBP or rGHBP tracer and a primary antibody raised in rabbits against rat GHBP (p(Ab)1)(Figures 6.2b and 6.2c). Guinea pig serum was, however, less effective than rat serum in displacing rGHBP tracer binding in the homologous rat GHBP radioimmunoassay (Figure 6.2c, Table 6.1). Guinea pig plasma and tissue also displaced the binding of ^{125}I-rGHBP to MAb 4.3 (Figure 6.3). GHBP immunoreactivity was present in comparable amounts in guinea pig (267 ± 46 ng/ml protein (n=6)) and rat (256 ± 49 ng/mg protein (n=6)) livers but was considerably more abundant in rat serum (2135 ng/ml) than in guinea pig serum (206.6 ± 55.7 ng/ml (n=7)). GHBP immunoreactivity, was not, moreover, detected in all guinea pig sera and the displacement of tracer binding was not parallel to that induced by the rGHBP standard or that induced by rat plasma or tissue extracts (Figure 6.3).

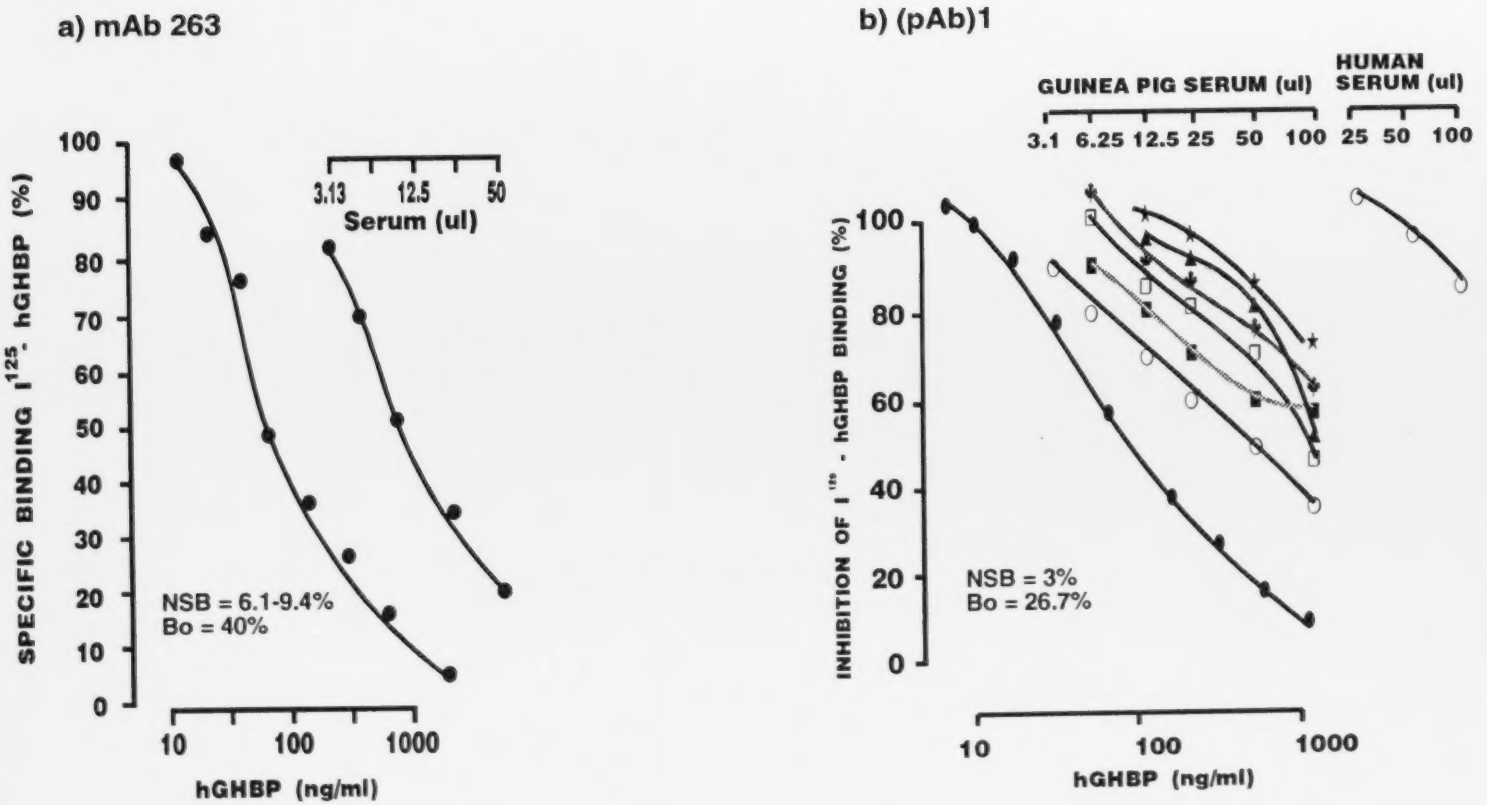

Figure 6.2. Cross-reactivity of individual guinea pig plasma samples in homologous and heterologous GHBP radioimmunoassays using monoclonal mAb 263 (a) or polyclonal antibodies (b and c) raised against the rat GHBP; in comparison with hGHBP (a and b) or rGHBP (c). Non-specific binding (NSB) and total tracer binding (Bo) are indicated for each assay.

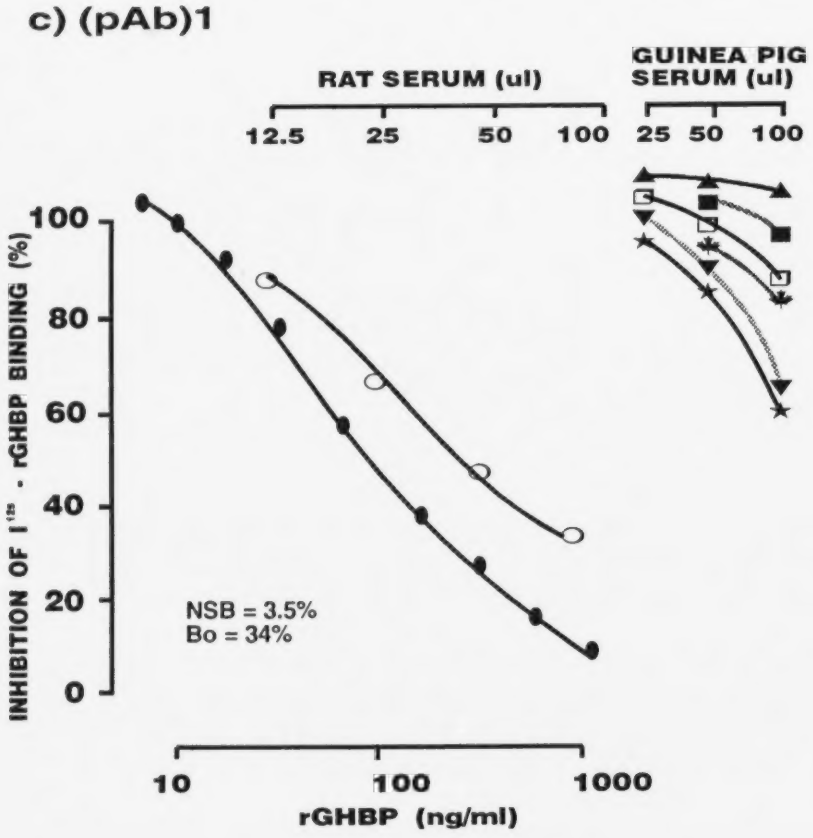

Figure 6.2., cont.

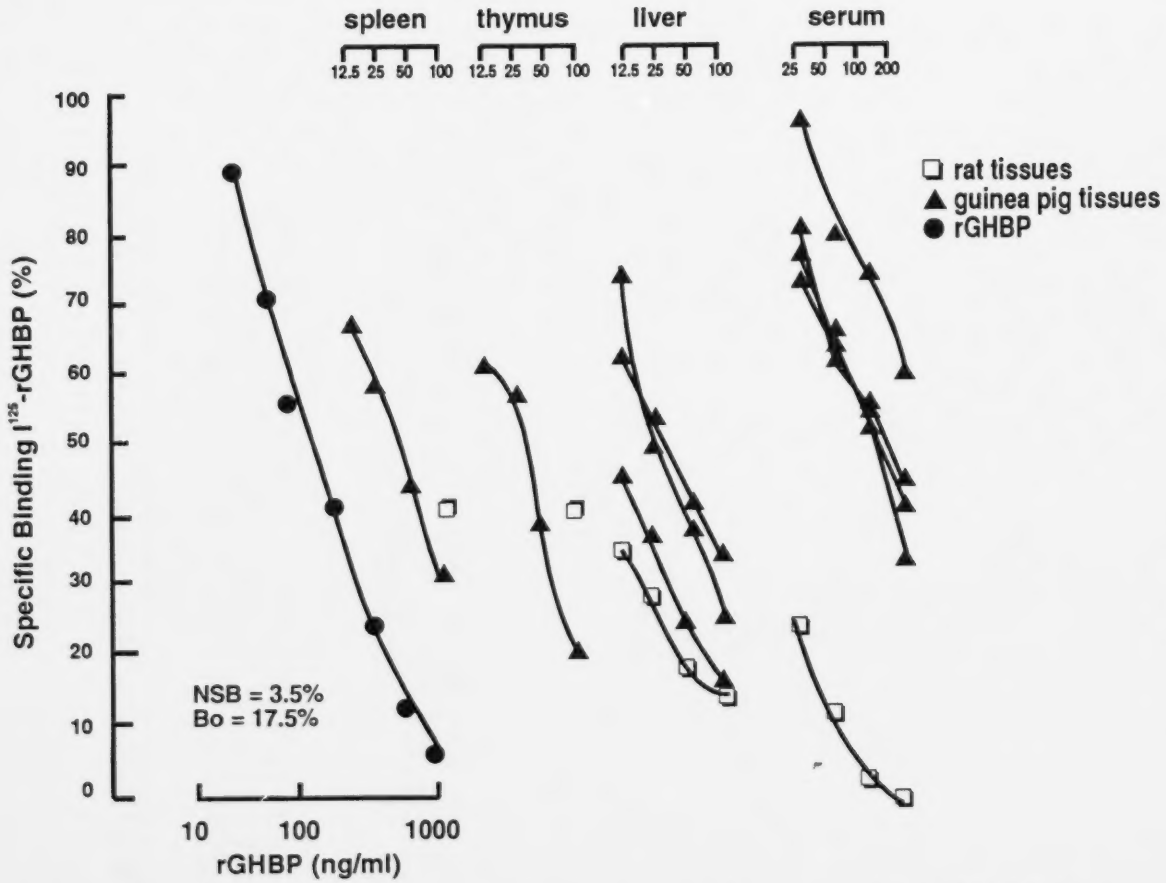

Figure 6.3. Cross-reactivity of guinea pig tissues and serum with MAb 4.3 in a radioimmunoassay using rGHBP as the standard and I^{125}-labelled tracer. The cross-reactivity of rat tissue extracts and serum is shown for comparison. Non-specific binding (NSB) and total tracer binding (Bo) are also indicated.

Table 6.1: Immunoreactive Plasma GHBP Concentrations

MAMMALS	mAb 263 IR ng hGHBP/ml	polyAb IR ng hGHBP/ml	polyAb IR ng rGHBP/ml
man	5-50	60	36
pig	5-30	50	ND
dog	ND	ND	ND
horse	5-30	50	ND
sheep	ND	50	ND
goat	ND	70	ND
cow	5-30	40	ND
rabbit	5-30	--	--
rat	5-20	300	240
mouse	--	106	76
guinea pig	>500 (n=9) (4672 ± 1117)*	170 ± 34* (n=9)	21 ± 8* (n=9)
BIRDS			
chicken	10-30		
REPTILES			
turtle	10-40		
AMPHIBIANS			
toad	10-30		
FISH			
flounder	70-100		
hagfish	50-100		
ratfish	50-150		
dogfish	50-70		

ND = not detectable
*mean ± S.E.M.'s

C. Western Analysis

The presence of GHBP/GHR immunoreactivity in guinea pig serum was also confirmed by Western analysis. GHBP immunoreactivity was first demonstrated in guinea pig serum and IgG-depleted guinea pig serum using polyclonal antibody 1 (p(Ab)1). A 46 kDa protein, larger than recombinant rGHBP, was specifically labelled (Figure 6.4a) that corresponded in size to the larger protein of the GHBP doublet in rat plasma. No labelling was observed if the primary antibody was omitted or replaced with pre-immune serum (data not shown) or was pre-incubated with rGHBP (Figure 6.4b).

Under similar conditions, immunoreactive GHBP proteins of 46 kDa and 40 kDa were also detected in rat plasma with another polyclonal antibody p(Ab)2 and with two monoclonal antibodies (MAb 263 and MAb 4.3) raised against rat GHBP (data not shown). GHBP immunoreactivity could not, however, be detected by these antibodies in Western blots of guinea pig plasma (data not shown). Nevertheless when excess p(Ab)2 or MAb 4.3 was used to precipitate proteins from guinea pig serum and extracts of guinea pig liver, a 46 kDa protein was readily labelled by MAb 263, as in rat liver and serum (Figure 6.5). The intensity of each of these immunoreactive bands was reduced by >80% when 2 µl rather than 20 ul of each sample was subjected to Western analysis. Larger proteins of 70 kDa were also occasionally observed in both rat and guinea pig serum and liver samples.

Guinea pig serum and rat serum were also immunoprecipitated with p(Ab)2 and immunoreactive proteins were detected by Western blotting with MAb 4.3, raised against the hydrophilic tail of the rat GHBP. A dose-related increase in the abundance of 46 kDa ($y = 0.016489x + 0.301774$; $r = 0.949147$, $p < 0.001(n=8)$) and 40 kDa ($y = 0.014914x + 0.237033$; $r = 0.912379$, $p < 0.001$ $(n=8)$) proteins was clearly evident in 1.25-80 µl rat serum (Figure 6.6). In contrast, a single, slightly larger protein of 48 kDa was visualized in guinea pig serum (Figure 6.7). The intensity of the signal detected increased with increasing serum volume ($r = 0.963593$, $p < 0.01$ $(n=5)$), although the equation of the regression line ($y = 0.001395x + 0.641597$) differed from that in rats.

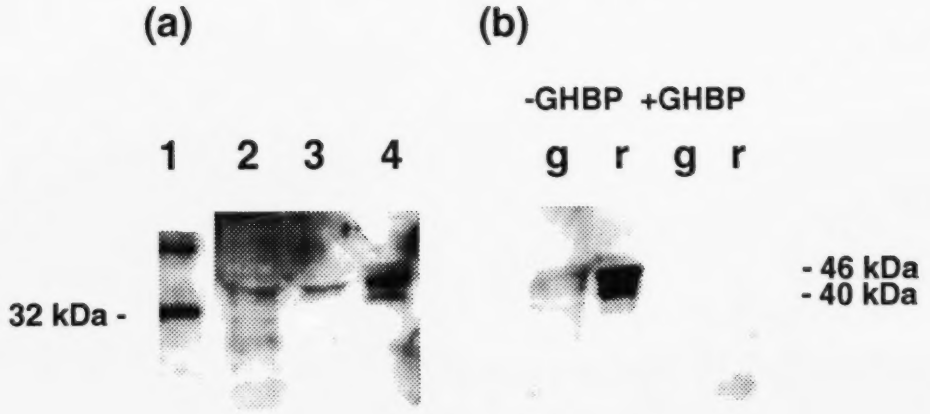

Figure 6.4. Western analysis showing proteins immunoreactive with p(Ab)1 antisera raised in rabbits against recombinant rat GHBP. (a) lane 1; recombinant rGHBP (7.5 ng); lane 2, IgG-depleted guinea pig serum (2 ul); lane 3, untreated guinea pig serum (2 ul); lane 4; untreated rat serum (1 ul). (b) untreated guinea pig (g) and rat (r) serum (2 ul each). Identical blots were stained with untreated p(Ab)1 (-GHBP) or with p(Ab)1 that had been preabsorbed with recombinant rat GHBP (+GHBP).

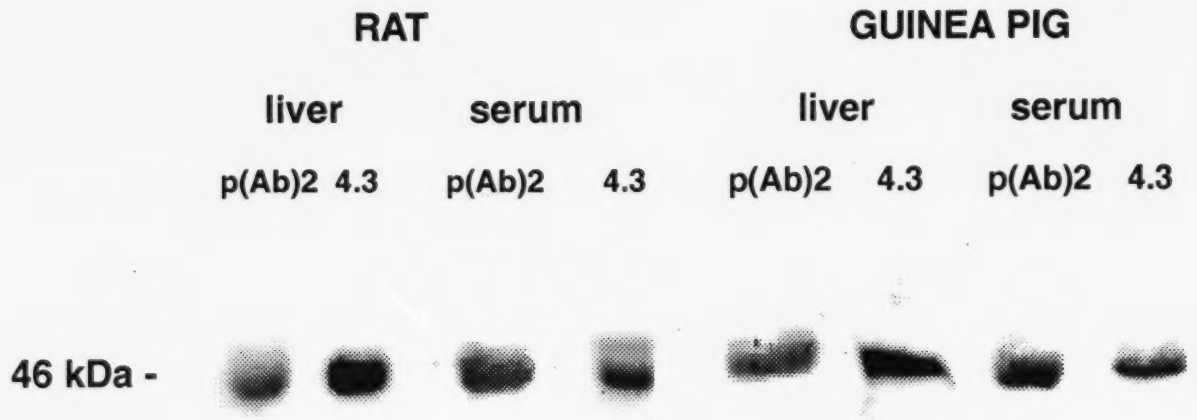

Figure 6.5. Western analysis showing proteins in rat and guinea pig liver and serum immunoreactive with MAb 263, raised against rat GHBP. 100 ul of serum or of liver homogenate was diluted to 1 ml in TBS buffer and incubated with 10 ul of a polyclonal antibody (pAb)2 raised against the rat GHBP or 10 ul of MAb 4.3 raised against the rat hydrophilic GHBP tail. Antibody-protein complexes were subsequently immunoprecipitated with protein A (Pansorbin), eluted with gel loading buffer and subjected to denaturing gel electrophoresis and Western transfer. Rat GHBP-like immunoreactivity was then detected using MAb 263 and visualized using HRP-labelled secondary antibodies and enhanced chemiluminescence. Larger bands of 70 kDa were also occasionally observed in rat and guinea pig serum and liver samples.

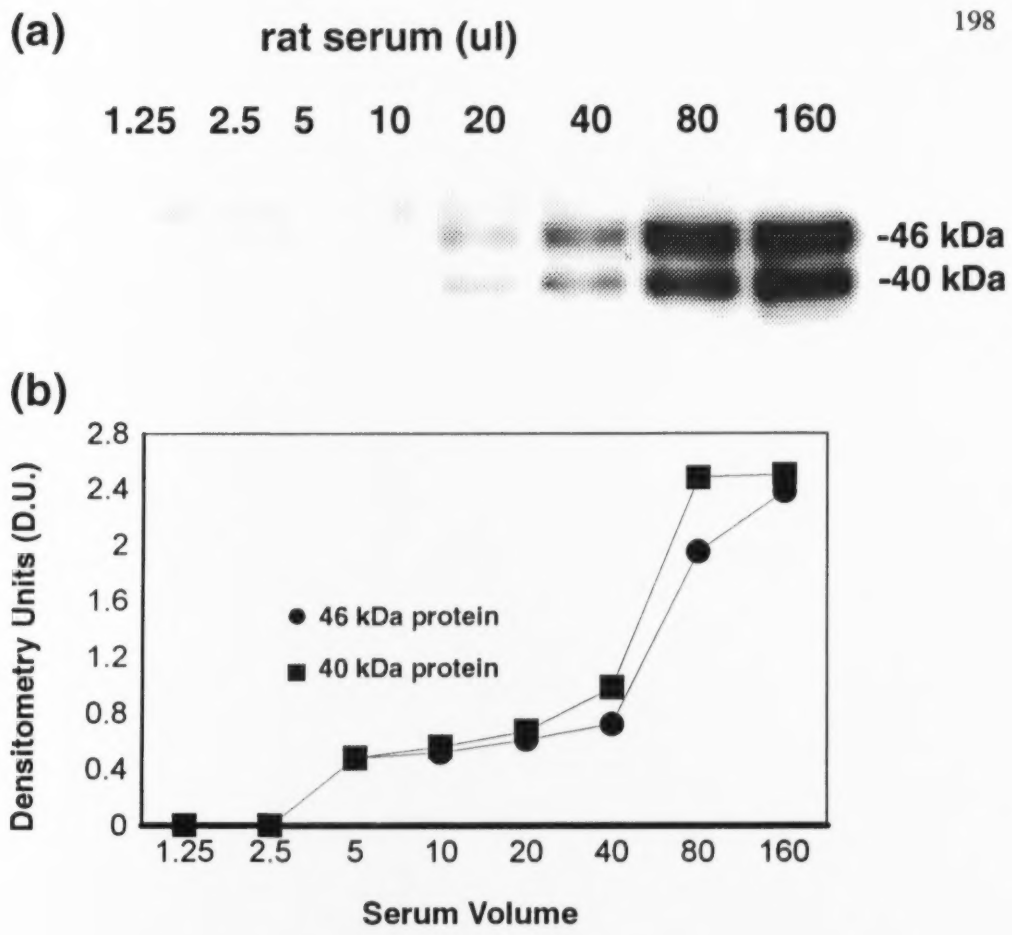

Figure 6.6. (a) Western analysis of rat serum showing proteins immunoreactive with MAb 4.3. Rat serum (1.25 - 160 ul) was diluted to 1 ml in TBS buffer and incubated with 10 ul of p(Ab)2 raised against the rat GHBP. Antibody-protein complexes were subsequently immunoprecipitated with Pansorbin and eluted with 50 ul gel loading buffer. Aliquots (15 ul) were then subjected to denaturing gel electrophoresis and Western transfer. Rat GHBP-like immunoreactivity was detected using MAb 4.3 and visualized using HRP-labelled anti-mouse antibodies and enhanced chemiluminescence. (b) The intensity of the signals detected were quantified by laser densitometry and expressed relative to serum volume. Increased serum volume was directly correlated with increased optical density ($r = 0.912379$ for the 46 kDa GHBP ($p < 0.001$), $r=0.949147$ for the 40 kDa GHBP ($p < 0.001$)). The slope of the curves for the detection of the 46 kDa and 40 kDa proteins were $y = 0.016489x + 0.301774$ and $y = 0.014914x + 0.237033$, respectively.

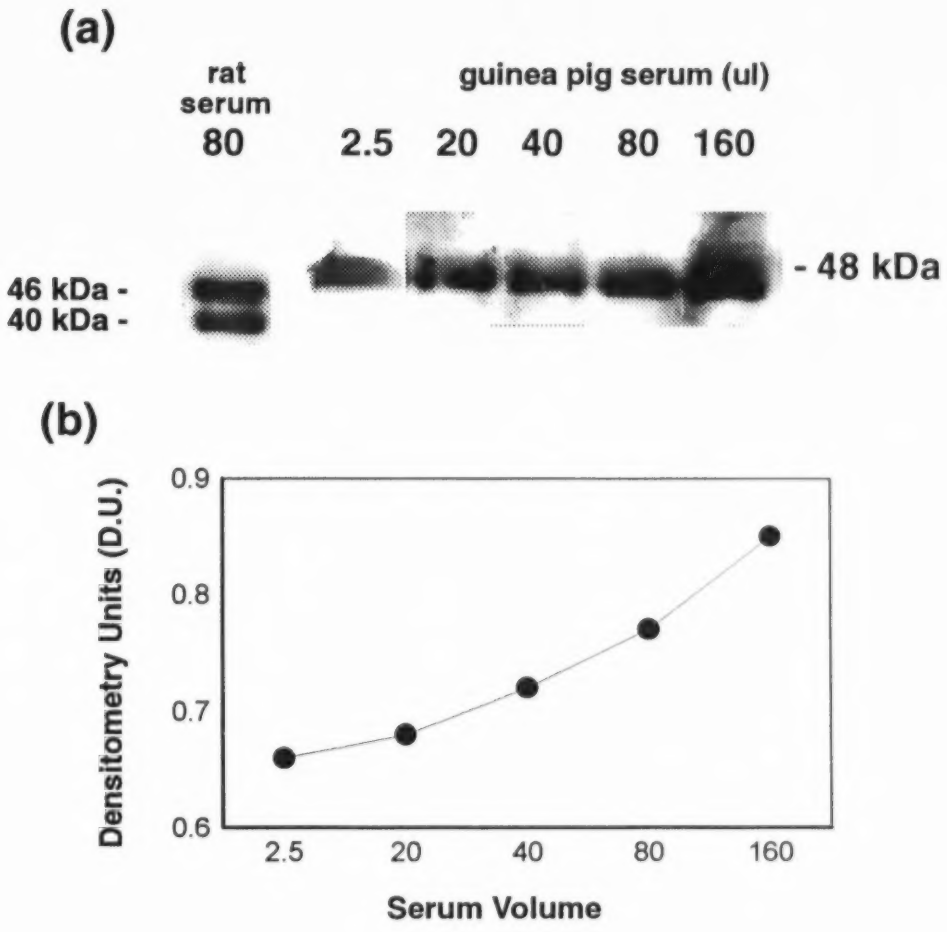

Figure 6.7. (a) Western analysis of guinea pig serum showing proteins immunoreactive with MAb 4.3. Guinea pig serum (2.5 - 160 ul) was diluted to 1 ml in TBS buffer and incubated with 10 ul of p(Ab)2 raised against the rat GHBP. Antibody-protein complexes were subsequently immunoprecipitated with protein A (Pansorbin) and eluted with 50 ul gel loading buffer. Aliquots (15 ul) were then subjected to denaturing gel eletrophoresis and Western transfer. Rat GHBP-like immunoreactivity was detected using MAb 4.3 and visualized using HRP-labelled anti-mouse antibodies and enhanced chemiluminescence. The position of immunoreactive GHBP bands in rat serum is shown for comparison. (b) The intensity of the signals detected were quantified by laser densitometry and expressed relative to serum volume. Increased serum volume was directly correlated with increased optical density ($r = 0.963593$ ($p<0.005$)). The slope of the curve for the detection of the 48 kDa band was $y = 0.001395x + 0.641597$.

Proteins with GHBP immunoreactivity, cross-reacting with MAb 263, MAb 4.3 and p(Ab)2 were also detected in guinea pig and rat liver (Figure 6.8). Although multiple proteins were labelled, proteins of 46 kDa were detected in both species. These 46 kDa proteins were not detected when the primary antibodies were replaced by PBS or pre-immune serum. Under the conditions used, preincubation of MAb 263 and p(Ab)2 with rGHBP diminished labelling of these hepatic 46 kDa proteins in rat and guinea pig liver by approximately 9.2% and 24.6% (n = 3), respectively. However, the preincubation of MAb 4.3 with the hydrophilic tail of the rGHBP completely suppressed labelling. In addition to 46 kDa proteins, larger guinea pig and rat liver proteins of 100 kDa cross-reacted with MAb 263 and p(Ab)2, whereas proteins of 92 kDa cross-reacted with MAb 4.3. These may reflect the presence of tissue GHRs or GHBP dimers, respectively. Preabsorption of these antibodies with rGHBP (MAb 263 and p(Ab)2) or with the hydrophilic GHBP tail (MAb 4.3) completely abolished the labelling of these proteins.

D. Radioligand Binding

Immunoprecipitation of I^{125}-hGH-incubated rat serum with p(Ab)1 indicated the presence of specific GH-binding proteins that bound 8.3% of the total radioactivity. Scatchard analysis indicated these binding sites had a binding affinity (Ka) of 2.85×10^{8} M^{-1} and a binding capacity (Bmax) of 2.9×10^{-10} . Immunoprecipitation of rat serum with MAb 4.3 also specifically precipitated 7.3% of the radioactivity incubated. Charcoal adsorption similarly demonstrated that radioactive hGH was specifically bound by rat (6%), rabbit (3.6%), dog (3.5%) and horse (2.4%) serums. In contrast, specific tracer binding to guinea pig serum could not be detected by immunoprecipitation, charcoal adsorption or gel filtration chromatography of I^{125}-oGH or I^{125}-hGH-treated guinea pig serum.

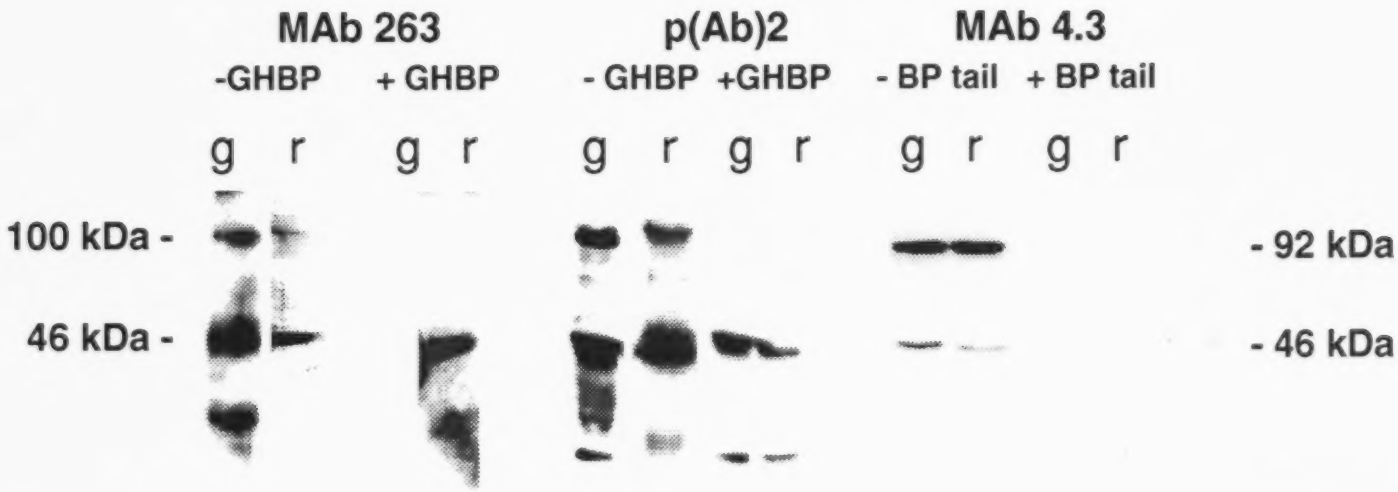

Figure 6.8. Western analysis showing the cross-reactivity of guinea pig (g) and rat (r) hepatic proteins with monoclonal (MAb 263 and MAb 4.3) and polyclonal (p(Ab)2) antibodies raised against the rat GHBP. Immunoreactive proteins were visualized using HRP-labelled second antibodies and enhanced chemiluminescence. The primary antibodies were (+) or were not (-) incubated with rGHBP or its hydrophilic tail (BP tail) prior to Western analysis.

6.4. DISCUSSION

Although Fairhall *et al.* (1992), Amit *et al.* (1992) and Daughaday *et al.* (1986) previously reported that guinea pigs lack serum GHBPs, these studies unequivocally demonstrate the presence of immunologically active GHBPs and GHRs in guinea pig serum and liver.

Northern Analysis of guinea pig and rat poly(A) RNA has confirmed the finding of Harvey and Fraser (1992) that the full-length, 4.4 kb GHR mRNA is present guinea pig liver. However, although Harvey and Fraser detected a single GHR transcript using a rabbit GHR cRNA probe, two mRNA species hybridized with a rat GHR cDNA probe in the present study. This is likely to reflect the greater phylogenetic relatedness of rats and guinea pigs. The additional, 1.9 kb transcript in guinea pig liver differs in size from the 1.2 kb mRNA encoding the rat GHBP. Despite this discrepancy, the smaller transcript in guinea pig liver may nevertheless code for a serum binding protein of comparable structure and size to the rat GHBP. Indeed, a GHR transcript of 2.6 kb has been identified in rat liver that encodes the structural components (including the hydrophilic tail) of the protein encoded by the 1.2 kb transcript (Tiong & Herington, 1991). This 2.6 kb rat liver transcript differs from the 1.2 kb transcript only in the length and composition of the 3' untranslated region and/or the length of the polyadenylated tail (Tiong & Herington, 1991).

Alternate gene transcription rather than proteolytic cleavage of membrane GHRs may thus provide a common origin for serum GHBPs in rodents instead of being a unique characteristic of rats (Sadeghi *et al.*, 1990) and mice (Baumbach *et al.*, 1989). However, the demonstration of a truncated GHR gene transcript is not, by itself, evidence for a guinea pig GHBP. Numerous GHR gene fragments have been detected in many mammalian and non-mammalian species (Herington *et al.*, 1991a; Herington *et al.*, 1991b; Hull *et al.*, 1993; Oldham *et al.*, 1993) that may be degradation products or code for proteins of unknown function. Indeed, Oldham *et al.* (1993) recently detected a 0.7 kb transcript in chickens that is not translated into a plasma GHBP. The 1.9 kb GHR mRNA in guinea pig liver may, however, be translated into a protein that resembles the GHBP in rat serum.

The GHBP in the serum of rats and mice has a hydrophilic tail that is not encoded by the full-length GHR transcript. The strong cross-reactivity of a guinea pig liver protein with MAb 4.3, raised against the hydrophilic tail of the rat GHBP, suggests that the GHBP in guinea pigs may similarly arise from alternate GHR gene expression.

In addition to sharing epitopes in the hydrophilic sequence of rat GHBP, the cross-reactivity of guinea pig serum awith MAb 263, p(Ab)1 and p(Ab)2 indicate that it also shares epitopes in the extracellular, ligand-binding domain of the GHR/GHBP. The immunoreactive GHBP in guinea pig serum also appears to be of comparable size (46 kDa) to the major GHBP in rat serum. However, while the GHBP immunoreactivity in guinea pig serum appeared to be solely due to a single protein, MAb 4.3 detected an additional moiety of 40 kDa in rat serum. The 46 kDa GHBP in guinea pig circulation may also differ structurally from that in rats. This possibility is supported by the greater cross-reactivity of guinea pig serum in the hGHBP RIA and poorer cross-reactivity in the rGHBP RIA (Figure 6.2) and by the difference between guinea pig and rat serum in the slope of the dose-response detection curves (Figures 6.6 and 6.7). The weaker staining of guinea pig serum proteins to the panel of antibodies used against rat GHBP may also indicate that guinea pig GHBP has a heterogenous structure of guinea pig GHBP's, although this may alternatively indicate a lower abundance of these proteins in guinea pig serum. The cross-reactivity of guinea pig serum with p(Ab)1 also suggests GHBP concentrations that are lower than those in rats, although present at concentrations higher than in many other mammalian species. Guinea pig serum was, however, found to have MAb263-detectable hGHBP-like moieties at concentrations far greater than those in rats or in other vertebrate species. These paradoxical findings may indicate differential cross-reactivity of different GHBP moieties with different GHBP antibodies. These results may, alternatively, merely reflect inherent vagaries in immunological detection and the poor compliance of antigen detection in different immunoassay systems and the presence of interfering factors in plasma (Sadeghi and Wang 1994).

Although GH resistance may result from a GHBP deficiency, the abundance of serum GHBP in guinea pigs is at least comparable with that in other mammalian species and is thus a null hypothesis for the etiology of GH-resistance in guinea pigs. Indeed, the

abundance of MAb 263 immunoreactivity in guinea pig sera may, conversely, suggest that an excess of serum GHBP may contribute to GH reistance in this species. This possibility is supported by the ability of GHBP to compete with membrane GH receptors for extracellular GH and to inhibit GH bioactivity *in vitro* (Lim *et al.*, 1990; Mannor *et al.*, 1991). An excess of serum GHBP may also induce chimeric dimerization with membrane GH receptors and thus impair the signal transduction mechanisms normally activated by GHR dimerization induced by GH binding (DeVos *et al.*, 1991; Wells *et al.*, 1993; Ilondo *et al.*, 1994). Overexpression of the serum GHBP has also been suggested to account for GH resistance in a rare form of human dwarfism (Duquesnoy *et al.*, 1993; Rieu *et al.*, 1993). While the dimerization of GHBP in serum has been hypothesized, it has, however, never been observed. The finding of a 92 kDa moiety in rat and guinea pig liver immunoreactive with MAb 4.3 may provide the first evidence for this phenomenon. Although these proteins were separated under denaturing conditions, denaturation may not be complete, as indicated by the dimeric appearance of the synthetic GHBP studies in these Western blots.

The immunoreactive GHBPs detected in guinea pig serum may not, however, be capable of binding GH. Indeed, the extreme pulsatility of serum GH levels in the guinea pig (Amit *et al.*, 1992) strongly suggests that GHBPs are defective in binding guinea pig GH, since GH:GHBP interactions dampen GH pulses in other species (Amit *et al.*, 1992). Although the binding of guinea pig GH was not assessed, the inability of radioiodinated human or ovine GH to bind guinea pig serum (Daughaday *et al.*, 1986; Fairhall *et al.*, 1992), as confirmed in the present study, may reflect a structural defect in the GH-binding region of the guinea pig GHBP. Such a defect could account for the poor cross-reactivity of guinea pig serum with some antibodies raised against the rat GHBP. The GHBP in guinea pig serum would thus appear to differ from the extracellular domain of the liver GHR, since GH-binding sites are abundantly present in hepatic membranes of these animals (Harvey & Fraser, 1992). Indeed the 100 kDa GHR immunoreactivity detected in guinea pig liver is likely to be due to the presence of membrane receptors. This strongly suggests that the serum GHBP in guinea pigs does not arise from the proteolytic processing of membrane GHR's and supports the possibility that it arises from alternate

processing of the GHR gene or from a separate GHBP gene. However, as the GHBP and GHR are co-regulated in other species and arise from the same gene or protein (Baumann & Shaw, 1988; Bingham *et al.*, 1994; Smith *et al.*, 1988; Baumbach *et al.*, 1989), these results suggest dysfunctional transcription of the GHBP gene in guinea pigs or a defect in post-transcriptional processing.

In addition to serum, GHBP immunoreactivity was also detected in rat and guinea pig liver, spleen and thymus. The liver may thus be the source of the 46 kDa GHBP found in guinea pig circulation, as hypothesized for rats (Smith *et al.*, 1988). The possible contribution of extrahepatic tissues to circulating GHBP concentrations is, however, unclear (Harvey& Hull, 1994). Although GHBPs may be present in extrahepatic tissues, they may be associated with cellular proteins and not be secreted in extracellular fluid (Barnard *et al.*, 1994; Frick *et al.*, 1994a; Frick *et al.*, 1994b).

In summary, these results clearly demonstrate the presence of GHBP-like proteins in guinea pig serum, which may be encoded by novel truncated GHR transcripts that are present in guinea pig liver.

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CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

7.1 SUMMARY OF RESULTS

These studies addressed the hypothesis that (a) GH unresponsiveness in tissues and (b) GH resistance in animals result from dysfunctional or absent GH receptors (GHRs).

A. Tissue GH Resistance

GH resistance at the tissue level was explored in two endocrine tissues, the thyroid gland and the pituitary gland, which have traditionally been considered to be unresponsive to GH. The conclusions of these studies are as follows:

1. The GH receptor gene is expressed in both somatotroph-rich and somatotroph-poor regions of the avian pituitary gland, suggesting both autocrine and paracrine roles for GH in pituitary function. GHR transcripts are also translated into functional receptors and/or binding proteins, as GH-binding moieties of approximately 60 kDa and 55 kDa are present in plasma and nuclear membranes of adenohypophyseal tissue and in pituitary cytosol, respectively. GH-binding moieties of 60 kDa and 55 kDa were also observed in hepatic plasma membranes and blood, respectively. Expression and translation of the GHR/GHBP gene also occurs in a rat somatolactotroph cell line (GH_3), in which GHBP-like immunoreactivity is primarily located in nuclear and cytoplasmic compartments. Thus, pituitary GH-resistance is not due to a deficiency of GH-binding activity. The abundance of truncated GHBPs not linked to signal transduction systems in GH_3 cells suggests that pituitary GH-binding sites may serve to protect the pituitary from excess GH stimulation. Alternatively, adenohypophyseal GHBPs may play a role in secretory function and contribute to the circulating GHBP pool *in vivo*, as immunoreactive GHBPs were present in media used for culturing pituitary explants and GH_3 cells.

2. The avian thyroid gland is similarly not deficient in GHR/GHBP transcripts or proteins. Moreover, this tissue is not totally GH-resistant, as thyroidal T_4 secretion is reduced by *in vivo* and *in vitro* GH administration.

B. Inherited GH Resistance

GH resistance with a genetic etiology was investigated in the sex-linked dwarf chicken and the guinea pig, as these animals are deficient in serum GHBPs and/or hepatic GHRs. The conclusions of these studies are as follows:

1. Despite a deficiency in hepatic I^{125}-oGH binding sites, expression of the GHR/GHBP gene was observed in SLD liver, brain, muscle, adipocytes and pituitary glands. Moreover, these transcripts were of normal size and composition, and were normally regulated ontogenically. Translation of the SLD GHR gene was also indicated by the detection of serum GHBPs and membrane GHRs of normal size in SLDs, and serum GHBPs capable of binding GH. Thus, the etiology of GH resistance in SLDs may involve defective post-translational processing of the GHR or a GHR point mutation but does not result from a GHR deficiency. The discrepancy between serum GHBPs and hepatic GHRs in their ability to bind I^{125}-oGH also suggests that the avian GHBP may arise from an alternate transcription product of the GHR gene.

2. GHBP immunoreactivity is abundantly present in guinea pig plasma, which is paradoxically deficient in immunoprecipitable proteins that bind radiolabelled hGH. The immunological detection of the hydrophilic sequence of rat GHBP in guinea pig liver and serum also suggests close structural homology with the C-terminal fragment of rat GHBP. Moreover, since this sequence is generated in rats from alternate transcription of the GHR gene, this result suggests guinea pig GHBPs may similarly arise from a truncated GHBP transcript identified in guinea pig liver. GH resistance in this species is therefore not associated with defective GHR gene expression or translation, but may result from abnormal receptor processing, dysfunctional signal transduction systems, or, putatively, an excess of serum GHBPs.

7.2 AVIAN GH RECEPTORS

A. Characterization

In the past ten years, the binding characteristics of avian hepatic GH receptors

have been extensively evaluated under various physiological and pathophysiological conditions. The hepatic GH receptor is considered to be of high affinity (Ka = 10-20 l/nmol) (Krishnan *et al.*, 1989; Gray *et al.*, 1991; Vanderpooten *et al.*, 1991a; Vanderpooten *et al.*, 1992) and low capacity (Bmax = 1.5-20 fmol/mg protein)(Krishnan *et al.*, 1989; Vanderpooten *et al.*, 1991a; Vanderpooten *et al.*, 1992), and receptor number rather than receptor affinity is altered by fasting and hormonal status (see section 1.4). This receptor is primarily somatogenic, as bovine GH (bGH) is a more effective ligand than cGH and prolactin is bound with low affinity, but lactogenic receptor populations may also be present in chicken hepatic membranes (Gray *et al.*, 1991). Hepatic GH-binding activity peaks transiently during embryonic growth, remains at low levels until 8 weeks post-hatch, and then increases until adulthood (Leung *et al.*, 1987; Vanderpooten *et al.*, 1991b). GHR mRNA follows a similar ontogenic pattern, but post-hatch GHR expression begins to rise 2 weeks post-hatch (Burnside & Cogburn, 1992).

The structural characteristics of avian GH receptors are, however, largely unknown. The studies described in Chapter 2, therefore, provide the first indication of the molecular size and sub-cellular distribution of hepatic and non-hepatic GHRs. Importantly, the GH-binding moieties identified in both hepatic and pituitary membranes were smaller than the calculated molecular mass (70 kDa) and the observed size (100 kDa) of mammalian GHRs, although smaller GH-binding moieties of approximately 60 kDa were detected in some earlier mammalian studies (see Table 1.2). Western analysis also detected a 55-60 kDa immunoreactive GHR in hepatic homogenates, although smaller (45 kDa) and larger (100-105 kDa, 70-75 kDa) moieties were also observed. Although this finding remains to be verified by other techniques and investigators, it may suggest that the absence of exon 3 in the cloned chicken GHR cDNA (Burnside *et al.*, 1991) could result in decreased receptor size. However, hepatic GHR mRNA is of similar size in avian and mammalian livers; thus, different translational or post-translational processing is more likely to account for this species variation.

These studies were amongst the first to demonstrate the presence of avian plasma GHBPs, although several initial reports (Jones *et al.*, 1990; Vasilatos-Younken *et al.*, 1991) were published during preparation of the manuscript. The 55 kDa GH-binding

protein detected in cytosolic and plasma samples (Chapter 2, Appendix 1) corresponds in size to the reported mammalian GHBP; however, it differs significantly from the 27.5 kDa and/or 69.5 kDa GHBPs detected by Vasilatos-Younken *et al.* (1991) in fresh serum using electrophoretic cross-linking techniques. The studies described in Appendix 1 also indicate the presence of a 70-75 kDa GHBP in avian serum (as detected by Western analysis). The 55 kDa protein initially detected in chicken serum (Chapter 2) may thus be a degradation product, since Vasilatos-Younken *et al.* (1991) found increasing amounts of a 52 kDa GHBP in chicken serum after storage, coincident with reduced amount of the 70 kDa GHBP. Similar results were also recently presented by Janssens *et al.* (1994), who found immunoreactive GHBPs of 70 kDa, 83 kDa and 140 kDa in chicken serum. The relationships between these GHBP isoforms and their physiological relevance remain unclear, although the chicken is undoubtedly not GHBP-deficient.

The origin of the avian serum GHBP remains unknown. Two mechanisms have been proposed to account for GHBP synthesis in mammalian species: alternate transcription of the GHR gene, or proteolytic cleavage of membrane receptors (Figure 1.6). The former mechanism is currently thought to occur only in rats and mice. However, the detection of multiple GHR transcripts (Chapters 2, 4 and 5) (Burnside & Cogburn, 1992; Huang *et al.*, 1993; Oldham *et al.* 1993) in avian hepatic and non-hepatic tissues suggests that the avian GHBP may also arise from alternate gene transcription. A GHBP-encoding transcript, however, has yet to be identified in this species, and truncated transcripts that do not always encode binding proteins have been detected (Oldham *et al.*, 1993). The possibility of divergent synthesis of GHBPs and GHRs is also supported by the finding of functional serum GHBPs, but not GHRs, in Cornell SLD chickens (Appendix 1).

B. Models of Laron-type Dwarfism

As described in section 1.5.C, Laron-type dwarfism (LTD) in humans is an inherited form of GH resistance associated with defective or absent hepatic GH-binding sites. However, the detection of GHR gene lesions and the development of therapeutic regimes and screening procedures in LTD patients have been complicated by the lack of

an animal model, as current mammalian models of dwarfism (eg. Snell dwarf mouse, lit/lit mouse) are associated with GH-insufficiency rather than GH-resistance. Indeed, potential therapeutic applications of SLD research were discussed in a recent symposium (Lessons from Laron Syndrome, Portugal, May 1992). The possibility of avian models of GH resistance was therefore investigated in Chapter 3.

SLDism in chickens and LTDism in humans are characterised by similar phenotypic derangements, including increased adiposity and reduced linear growth. The endocrine profiles in the two syndromes are also homologous, as plasma concentrations of bioactive GH are normal or elevated, despite low or absent plasma IGF-I levels and hypothyroidism. LTD and SLD may share a common etiology, as hepatic GH-binding is diminished in at least some LTDs and SLDs (Leung *et al.*, 1987). The difference in GH-binding between SLDs/LTDs is most apparent in adults (Vanderpooten *et al.*, 1991b). Analysis of the GHR gene of different populations of LTD patients has detected a panel of deletions, point mutations and nonsense mutations (Figure 1.4). However, prior to 1991, the SLD GHR gene had not been subjected to a similar scrutiny. The identification of a 1.0-1.7 kb deletion in the GHR gene of a Connecticut strain of broiler SLDs by Burnside *et al.* (1991) was therefore the first indication that SLDs and LTDs may indeed share a common etiology. However, as shown in Chapter 3, GHR mRNA in the Cornell strain of SLDs is of normal size, and no point mutations are present in the portions of cDNA sequenced. Thus, SLDism in chickens, like LTDism in humans, is a heterogenous family of GHR lesions and may indeed provide an experimental model of this human disease.

Recent analysis of the GHR in populations of LTDs and SLDs has now identified additional gene lesions. For instance, the GHR gene is present and of normal size in SLD fowl strains obtained from University of Georgia flocks(GA), but the gene is not transcribed. The GHR gene in these birds may thus have an abnormal promoter region (Burnside *et al.*, 1992). Abnormalities in ontogenic regulation of GH-binding sites may also result in dwarfism, as hepatic GH-binding activity increases post-natally in normal but not SLD chickens of the Belgian (Vanderpooten *et al.*, 1991b) and Hubbard (Leung *et al.*, 1987) SLD strains and in normally-growing humans but not in GH-resistant

pygmies (Merimee, 1992). However, abnormal regulation of GHR transcription is not a universal finding in GH-resistance, as GHR mRNA increases with age in both SLD and normally-growing (K) Cornell chickens (Chapter 5). Abnormal transcript splicing has also been shown to result in the production of nonfunctional GHRs in both SLDs (Huang *et al.*, 1993) and LTDs (Maiter *et al.*, 1989; Amselem *et al.*, 1993).

GH resistant syndromes are, however, also associated with subpopulations characterised by normal GHR expression (Chapter 5) and high levels of immunoreactive GHBPs and GHRs. For instance, in the Cornell strain of SLDs, GHR mRNA is of normal size (Chapter 5) and immunoreactive GHRs/GHBPs are present in liver and serum that correspond in size to proteins in normally-growing chickens (Appendix 1). Similarly, Northern analysis and immunocytochemistry of fibroblasts from four unrelated LTD patients also revealed GHR mRNA of normal size and immunoreactive plasma membrane GHRs (Postel-Vinay *et al.*, 1991). Immunoreactive and bioactive GHBPs are also present in numerous LTD patients (Maheshwari *et al.*, 1992; Savage *et al.*, 1993), and may be present in the Cornell strain of SLDs (Appendix 1). Defective signal transduction may therefore account for impaired growth in these populations. In support of this hypothesis, a point mutation was recently identified in the GHR gene of several GHBP-positive LTD patients that have normal membrane expression of GHRs (Duquesnoy *et al.*, 1993; Rieu *et al.*, 1993). Expression of this mutant in COS-7 cells results in proteins that are capable of binding GH but that are incapable of dimerization, as the point mutation lies at a point of interface between the two receptor molecules (Rieu *et al.*, 1993). Intracellular mutations may also result in decreased efficiency of signal transduction and varying degrees of growth impairment. Accordingly, 2 point mutations were recently identified in the GHR gene of a Laron dwarf (Kou *et al.*, 1993). However, the signficance of this finding is unknown, as both the patient and his mother were heterozygous for these mutations and the mother was not growth-deficient.

Thus, the studies presented in Chapter 5 and Appendix 1 have provided evidence in support of the heterogeneity of sex-linked dwarfism and may provide insight into similar LTD populations. These studies also provide critical information regarding the structural requirements for GH binding and action.

7.3 IMPLICATIONS

A. Evolving Concepts in GHBP Roles

i. Pituitary GHBPs: Since the initial reports of pituitary GH-binding activity (Chapter 3), numerous reports have been published extending these observations. The specific localization of GHR/GHBP immunoreactivity in different pituitary cell types and within various intracellular compartments is of special interest. In the initial studies of Fraser and Harvey, (1992), GHBP immunoreactivity and mRNA was located ubiquitously in GH-secreting and non-GH secreting cells of the rat pituitary, which corresponds to the localization of GHR mRNA in the caudal and cephalic lobes of the chicken pituitary (Chapter 3). The ubiquitous nature of adenohypophyseal GHBPs was underlined by a subsequent study, in which GHBPs were co-localized with GH, PRL, LH, FSH, TSH and ACTH in their respective cell types (Harvey *et al.*, 1993). However, Mertani *et al.* (1994) observed specific GHBP immunoreactivity in somatotrophs, lactotrophs and gonadotrophs but not in thyrotrophs or corticotrophs. Moreover, the lack of radioiodinated GH uptake in thyrotrophs and corticotrophs further supports the hypothesis of cell-specific GHR/GHBP expression (Mertani *et al.*, 1994). The cause of the discrepancy between cellular patterns of immunoreactive GHBPs described by Harvey and co-workers (Fraser & Harvey, 1992; Harvey *et al.*, 1993) and Mertani *et al.* (1994) is unclear, but may relate to the use of different antibody concentrations and/or different fixation methods. In any case, the localization of GHBP/GHRs in somatotroph and non-somatotroph cell populations is suggestive of autocrine and paracrine roles of GH within the pituitary gland. Paracrine and autocrine roles for prolactin have also recently been identified in GH_3 cells, as prolactin stimulates PRL and GH secretion from these cells and also stimulates cell proliferation (Krown *et al.*, 1992).

The intracellular distribution of pituitary GHBPs is similarly controversial, as Harvey and co-workers (Fraser & Harvey, 1992; Harvey *et al.*, 1993) detected high GHBP levels in secretory granules with numerous antibodies and nuclear staining with MAb 263 but not with p(Ab)1 nor MAb 4.3. Mertani *et al.* (1994), conversely, detected immunoreactive GHBPs in the cytoplasmic matrix, nuclear euchromatin and nuclear membranes but not in secretory granules. Nuclear GHBP immunoreactivity was similarly

observed in GH_3 cells (Chapter 3). Resolution of this discrepancy would provide considerable insight into the possible roles of the pituitary GHR/GHBP. The possibility of some form of GH autoregulation is discussed in detail in the introductions to Chapters 3 and 4, and is also supported by the somatotroph hypoplasia and reduced pituitary GH production in mice transgenic for the bGH gene (Stefaneanu *et al.*, 1993; Aulakh *et al.*, 1994). However, the inability of many investigators to demonstrate ultrashort loop regulation of GH secretion suggests that the pituitary GHR/GHBP is involved in processes other than regulation of GH secretion. For instance, the localization of GHBPs in secretory granules coupled with the observation of GHBP secretion from GH_3 cells is suggestive of a role for GHBPs in secretory processes. The GHBP may protect GH from intrapituitary degradation or selectively target GH to various storage pools. The cytoplasmic GHBPs detected in avian (Chapter 2) and mammalian pituitaries and in pituitary cell lines (Chapter 3) may also modulate GH secretion, but they may also inhibit GH action by competing with signal-mediating receptors. Evidently, pituitary GH levels greatly exceed serum concentrations, thus this buffering system may be necessary to protect the pituitary gland from excessive GH stimulation. Alternatively, as suggested by the uptake of radioiodinated GH by pituitary cells (Stern *et al.* 1976; Mertani *et al.*, 1994), cytoplasmic GHR/GHBPs may simply be indicative of internalized receptors.

The studies described in Chapter 3 also challenge current theories involving the source of the circulating GHBP. Prior to this report, secreted GHBPs were observed in the media of cultured rat hepatocytes (Barnard *et al.*, 1994) and a human hepatoma cell line (Amit *et al.*, 1994) but not in the media of cultured rat adipocytes (Frick & Goodman, 1992) or mouse trophoblasts (Barnard *et al.*, 1994), thus suggesting a purely hepatic site of origin. As GHBP mRNA and intracellular GHBPs are abundantly present in adipocytes and trophoblasts, it has been hypothesized that differential glycosylation patterns between hepatically-produced and nonhepatically-produced GHBPs may result in GHBP secretion (liver) or retention (other tissues) (Frick *et al.*, 1994). However, the presence of immunoreactive GHBPs in media conditioned by a variety of tissues, including pituitary cells, (Figures 3.8) is suggestive of a more widespread distribution of GHBP-secreting cells. Indeed, total amount of circulating GHBPs is greater than that of

GHRs in rabbit liver membranes (Ymer & Herington, 1985); thus, non-hepatic sources of circulating GHBPs are probable.

B. Evolving Concepts in GH Resistance

GH resistance may not be a solely pathophysiological phenomenon, but may also serve a physiological role in the modulation of hormonal action. Traditionally, the distribution of hormone receptors was thought to be limited to specific target glands, and consequently the presence of receptors or receptor transcripts was thought to signify tissue responsiveness. However, the ubiquitous nature of the GHR detailed in this thesis and by others (Barnard *et al.*, 1991; Harvey & Hull, 1994) challenges this dogma, as some actions of GH are not similarly ubiquitous. For instance, GH induces a proliferative response in most tissues (eg. gastric mucosa, bone) but not in thyroid glands (Mayerhofer *et al.*, 1990; Lobie *et al.*, 1992). However, GH-binding activity is abundantly present in this tissue, and GH exposure results in a modification of thyroidal T4 synthesis and/or secretion (Chapter 5). Thus, it could be said that this tissue is selectively resistant to GH. Similarly, the pituitary gland in most mammalian and avian species other than bovines is resistant to the autoregulatory actions of GH despite the presence of abundant GH-receptor transcripts and proteins. Interestingly, the responsiveness of the bovine pituitary to GH feedback may be related to the greater abundance of mammosomatotrophs (accounting for all acidophil GH-secreting cells) in cow pituitary glands, since tumorous somatolactotrophs are also responsive to GH autoregulation. In contrast, somatolactotrophs only comprise 8-15% of the GH-secreting cells in rat pituitary glands (Harvey, 1994).

Thus, tissue responsiveness is not solely determined by the presence of receptors and may be selective for specific cellular responses. This specificity may arise at the level of signal transduction. Indeed, cellular specificity of GH signal transduction systems and of proteins associated with the GHR has been described in Chapter 1. Differential synthesis of the GHR and GHBP may also regulate GH responsiveness in a tissue-specific way, as GHBP mRNA predominates in some tissues (eg. liver) whereas the full-length transcript is the major form in spleen and muscle (Bingham *et al.*, 1994). Preferential

production of GHBPs rather than full-length GHRs would inhibit GH action by competing with signal-transducing receptors for available ligand. Alternatively, GHBPs may modulate rather than inhibit GH action by forming heterodimers with full-length receptors. Although speculative, it is possible that GHBP-GHR dimers may be not be biologically inert, but may be capable of activating the same or different signal transduction pathways as GHR-GHR homodimers.

Moreover, this concept of selective resistance is also observed in numerous pathophysiological conditions. For instance, generalized resistance to thyroid hormone (GRTH) results from defective β thyroid hormone receptors and is characterized by high T3 levels (Refetoff *et al.*, 1993). However, the α T3 receptor is unaffected in this disorder, thus tissues containing a predominance of α receptors would manifest hyperthyroid symptoms, whereas β receptor-containing tissue would be thyroid deficient (Refetoff *et al.*, 1993). GH-resistance in humans may also be somewhat selective, as Laron dwafism affects somatogenic but not lactogenic receptors. For instance, immortalized T-cells isolated from LTD patients do not proliferate in response to GH. However, GH can induce metabolic effects such as insulin resistance in these GHR-deficient cells via prolactin receptors, albeit at concentrations ten-fold greater than those required in normal cells (Geffner *et al.*, 1993).

The studies described in this thesis also propose a novel cause of GH resistance, both in individual tissues and in GH-resistant syndromes, as the GH-resistance in the pituitary gland and in the guinea pig may result from excess GHBPs. GHBPs inhibit GH action *in vitro* by competing with GHRs for available ligand and by inhibiting GHR dimerization (Baumann, 1994; Dattani *et al.* 1994; Harvey & Hull, 1994a). However, the *in vivo* effect of GHBPs is thought to be stimulatory, as co-administration of GHBPs in therapeutic GH regimens enhances the the growth response (Harvey & Hull, 1994a). However, in pathophysiological levels such as those observed in the guinea pig, the GHBP may competitively inhibit GH action. High levels of serum GHBP would reduce the proportion of free GH capable of interacting with target site GHRs, and extracellular and intracellular GHBPS could compete with receptors for available GH. This hypothesis is supported by several clinical reports that were published during the course

of these studies (Duquesnoy *et al.*, 1993; Rieu *et al.*, 1993). The patients described in these reports exhibited some symptoms of GH-resistance (short stature, elevated plasma GH) despite the presence of functional GHBPs. However, Scatchard analysis of this serum GH-binding activity revealed a binding capacity 30-100 fold greater than normal, thus pathophysiological GHBP levels may indeed play an inhibitory role in GH action.

7.4. CONCLUSIONS

These results may provide insight into clinical syndromes of GH unresponsiveness and a greater understanding of the roles of GH, GHBPs and GHRs in target tissues and their interactions in physiological and pathophysiological states. This thesis also emphasizes the heterogeneity of lesions that may result in GH resistance. Indeed, a variety of defects in GHR/GHBP gene expression have been identified and implicated in genetic states of GH resistance. A receptor deficiency is not, however, axiomatically responsible for hormone resistance, which may conversely result from overexpression of truncated GHBPs and their competition with membrane receptors for available ligand. Furthermore, these studies also demonstrate that GH resistance can occur despite normal GHR/GHBP expression, indicating the importance of membrane phenomena (eg. receptor insertion, configuration, and dimerization) and intracellular mediators (signal transduction systems) that are normally required for hormonal action. GH resistance may thus result from a variety of defects and consequently generalities about GH resistance are impossible and absurd. Indeed, it is now recognized that pathological and genetic states of GH resistance (such as Laron-type dwarfism and sex-linked dwarfism) do not have a single etiology and reflect numerous subsets rather than homogenous populations. Within individuals, moreover, GH resistance may be hereditary or pathological or physiological and occur at multiple extracellular and cellular loci. Tissue resistance may result from defects at post-translational levels and could occur ubiquitously or be tissue-specific. Some of these putative sites of GH resistance are indicated in the model shown in Figure 7.1.

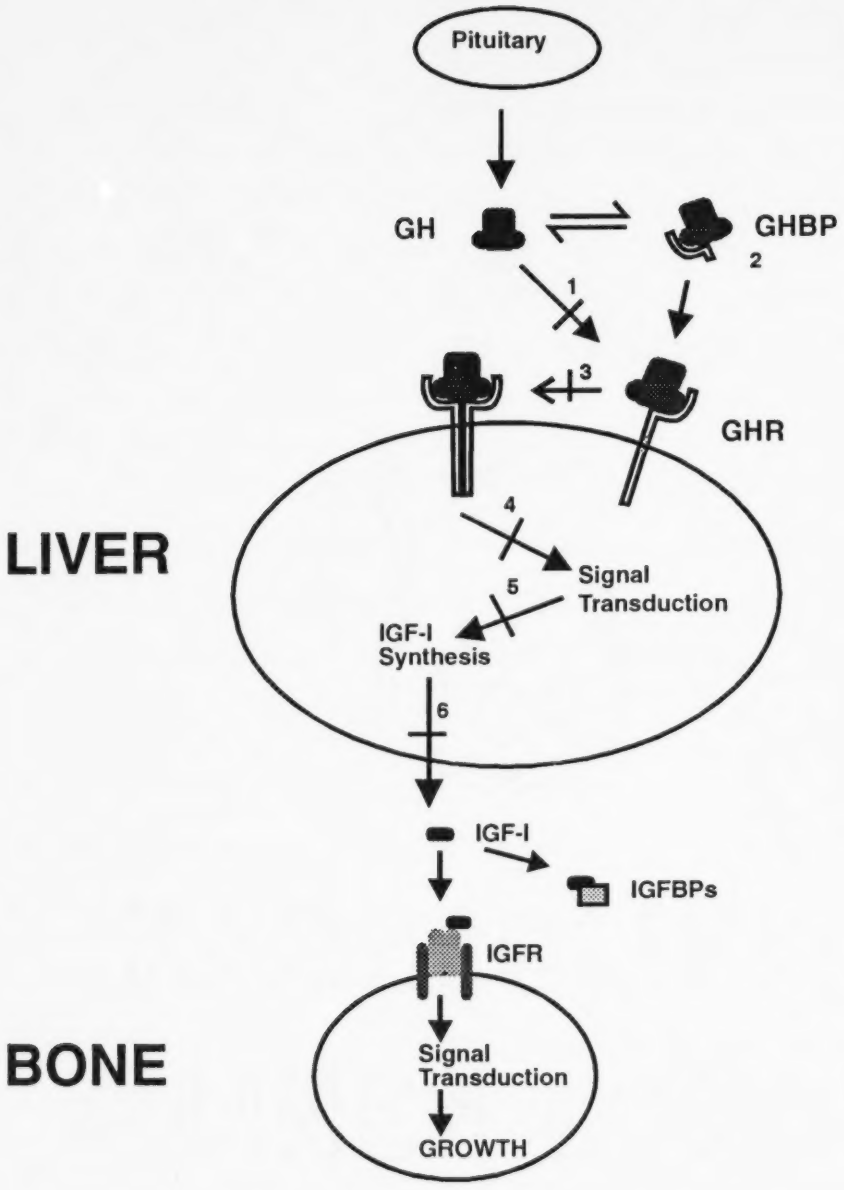

Figure 7.1. Potential defects capable of resulting in growth hormone (GH) resistance: 1) Abnormalities in the binding domain of growth hormone receptors (GHR); 2) An excess of serum GHBPs; 3) Defective receptor dimerization; 4) Abnormal signal transduction, resulting from a defect in the intracellular domain of the GHR; 5) Defect in IGF-I synthesis; 6) Defect in IGF-I secretion. Abbreviations: growth hormone (GH); growth hormone binding protein (GHBP); insulin-like growth factor-1 (IGF-I); insulin-like growth factor binding proteins (IGFBP); insulin-like growth factor receptor (IGFR). (Adapted from Rosenfeld et al., 1994).

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APPENDIX 1: TRANSLATION OF THE GH RECEPTOR/GH-BINDING PROTEIN GENE IN SEX-LINKED DWARFS

A1.1 INTRODUCTION

The critical role of growth hormone (GH) in growth has long been recognized and dwarfism results from a GH deficiency. Dwarfism can, however, also result from GH resistance, in which GH concentrations are elevated but tissues are unresponsive to GH action. Sex-linked dwarfism in poultry, for instance, is characterized by a 30-40% reduction in body weight (Guillaume, 1976; Tixier-Boichard *et al.*, 1989; Decuypere *et al.*, 1991) even though these birds have normal or elevated concentrations of bioactive GH in their plasma (Harvey *et al.*, 1984). Sex-linked dwarfism is, thus, due to GH resistance, especially as GH-dependent lipolysis, monodeiodinase activity and IGF-I production are impaired and not enhanced by exogenous GH (Harvey *et al.*, 1984; Bowen *et al.*, 1987; Huybrechts *et al.*, 1987; Leung *et al.*, 1987; Kuhn *et al.*, 1990). Indeed, sex-linked dwarfism is correlated with diminished binding of labelled GH to target tissues, as a result of GH receptor (GHR) dysfunctions (Leung *et al.*, 1987; Vanderpooten *et al.*, 1991).

The GHR gene has been characterised in numerous SLD strains, and gene deletions and defective gene transcription or post-transcriptional processing account for the GH resistance of some strains (Burnside *et al.*, 1991; Burnside *et al.*, 1992; Huang *et al.* 1993). Sex-linked dwarfism is, however, also associated with normal transcription of the GHR gene (Hull *et al.*, 1992). Studies in the Cornell strain of SLDs have, for instance, revealed multiple GHR gene transcripts of 4.4 kb, 2.8 kb and 1.0 kb in normally-growing (K) and SLD chickens, and sequencing of most of the coding domain did not reveal any point mutations or deletions (Hull *et al.*, 1992). Defects in GHR translation or post-translational processing may therefore account for GH resistance in these birds. This possibility was therefore examined by determining if hepatic GH-binding proteins (GHBPs) were present in Cornell dwarfs.

Soluble or serum GHBPs arise from cleavage of the extracellular domain of the membrane GHR or from alternate transcription of the GHR gene (Harvey & Hull, 1994). Circulating GHBPs in rats and humans are thus absent or minimally present in

physiological or pathophysiological states associated with GH resistance. Although circulating GHBPs have been detected in normally growing birds (Vasilatos-Younken *et al.* 1991; Zhong *et al.* 1994) it is unknown if they are present in SLD chickens.

A1.2. MATERIALS AND METHODS

A1.2.A. Western Analysis

The possibility that SLD serum and liver may be deficient in immunoreactive GHBP/GHR or contain GHBPs or GHRs of anomolous size was evaluated by Western Analysis. Aliquots of 1-2 µl of serum or 100 µg of liver tissue (homogenized in 1% w/v sodium dodecyl sulfate (SDS) containing 1 mM PMSF (Sigma) and 10 µg/ml aprotinin (Sigma)) were diluted in gel loading buffer (62.5 mmol/l Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.02% (w/v) bromophenol blue), subjected to electrophoresis in 15% acrylamide(ICN, Aurora, OH)-SDS gels, and subsequently electroblotted onto Immobilon P membranes (Millipore, Mississauga, Ont.) in 10 mmol/l 3-[cyclohexylamino]-1-propane-sulfonic acid buffer (Sigma), pH 10, containing 10% (v/v) methanol. The filter was incubated overnight with a polyclonal antibody raised against recombinant chicken GHBP (p(Ab), American Cyanamid, 1:2000) in Tris-buffered saline (TBS) (10 mmol/l Tris (pH 7.5), 150 mmol/l NaCl) containing 5% (w/v) non-fat dried milk following a 1 h blocking step in the same buffer. A polyclonal antibody raised against a putative membrane-bound chicken GHBP (CH17) (American Cyanamid)(1:2000) was similarly employed for the screening of identical Western blots. This protein was expressed by a chicken GHR cDNA clone obtained from a avian hepatic library, and contains the extracellular and transmembrane domains of the GHR and a portion of the intracellular domain (Bingham *et al.* 1994). Replicate blots were incubated in the absence of primary antibody or with antibody that had been pre-absorbed with an excess of CH17 for 1 h. The blot was subsequently washed in TBS (4 x 30 min) and incubated with horseradish peroxidase-labelled goat anti-rabbit IgG (Amersham, Lancashire, England) for 1 h at a dilution of 1:1500. Following four 30 min washes in TBS containing 1% (w/v) bovine serum albumin (BSA, Sigma), the filter was developed using a ECL kit (Amersham) and autoradiographed.

A1.2.B. Binding Studies

The possible translation of the GHR message was qualitatively assessed by determination of GH-binding sites in K and SLD serum. Aliquots (1 ml) of serum were incubated for 2 h at 39°C with approximately 1.3 x 10^6 cpm ^{125}I-labelled (specific activity 40-50 μCi/μg) ovine GH (oGH-14, biological grade, 1.4 IU/mg, NIADKD, Bethesda, MD, USA), (Fraser *et al.* 1990). The tracer was >95% precipitable with excess 10% (w/v) trichloroacetic acid and eluted as a single monomeric peak when chromatographed (Harvey & Scanes, 1977) on Sephadex G-100 (Pharmacia Fine Chemicals). The tracer was used within 4 - 5 days of iodination with fresh Na^{125}I (Edmonton Radiopharmaceutical Centre, Edmonton, Canada). The separation of free and bound GH was achieved at 4°C by gel filtration of the incubation media on 1x50 cm columns of Sephadex G-100 in a 10 mmol/l sodium phosphate elution buffer (containing 10 mmol/l EDTA, 150 mmol/l NaCl, 0.1% BSA (w/v), 0.1% (w/v) sodium azide and 20% (v/v) glycerol, pH 7.6), at a flow rate of 1.0 ml/min. (Snow, Shaw, Winer & Baumann, 1990). Fractions (1 ml) were collected and aliquots (100 ul) were counted for radioactivity. The radioactive peaks were characterized by comparison with ^{125}I-labelled oGH and Na^{125}I and with the elution of molecular weight (MW) markers (blue dextran, MW = 2000 kDa, aldolase, MW = 150 kDa, collagenase, MW - 105 kDa, BSA, MW - 66 kDa, carbonic anhydrase, MW = 29 kDa, and cytochrome c, MW = 12 kDa, Sigma Chemical Company, St. Louis, Mo, USA) that were monitored spectophotometrically at 280 nm.

A1.3. RESULTS

A1.3.A. Western Blotting

The presence of GHBP/GHR immunoreactivity in K and SLD liver and serum was demonstrated by Western Analysis. GHBP immunoreactivity was first demonstrated in serum using a polyclonal antibody raised against recombinant chicken GHBP (cGHBP) (p(Ab)GHBP). A 74 kDa protein was labelled in SLD serum that corresponded in size to a protein present in K serum (Figure A1.1). A larger protein of 143 kDa was also occasionally detected in the serum of both strains. No labelling was observed if the

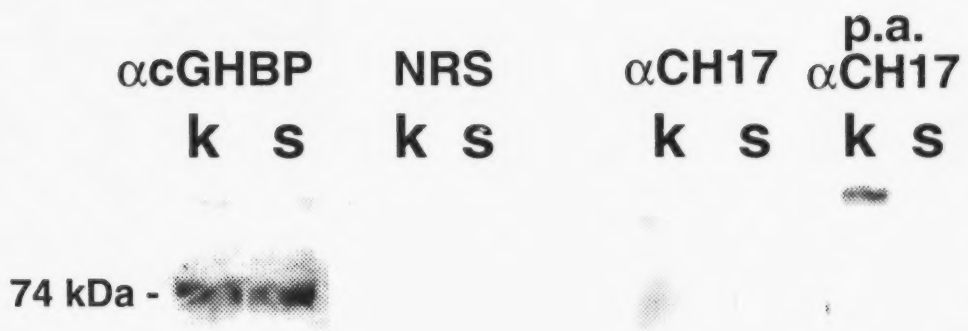

Figure A1.1. Western analysis showing the cross-reactivity of normally-growing (K) and sex-linked dwarf (S) serum proteins with polyclonal antibodies raised against recombinant chicken GHBP (αcGHBP) or against CH17 (αCH17). Immunoreactive proteins were visualized using HRP-labelled second antibodies and enhanced chemiluminescence. Identical blots were also incubated with αCH17 that had been pre-absorbed with CH17 (p.a. αCH17) or with normal rabbit serum (NRS).

primary antibody was omitted (data not shown) or replaced with pre-immune serum (Figure A1.1). Sera were also screened with a polyclonal antibody raised against a chicken GHR-like peptide (CH17) (p(Ab)CH17) which contains the extracellular and transmembrane domains and a portion of the intracellular domain (Bingham *et al.* 1994). However, this antibody did not detect any proteins in either K or SLD serum.

GHBP-like immunoreactivity was also present in hepatic homogenates (Figure A1.2). Proteins of 55 kDa, 70-75 kDa, 100-105 kDa and 200 kDa found in K and SLD liver cross-reacted with p(Ab)GHBP. With the exception of the 200 kDa moiety, the GHBP-like proteins did not cross-react with preimmune rabbit serum (Figure A1.2). p(Ab)CH17 also detected a protein of approximately 55 kDa in K and SLD homogenates, however, an additional 64 kDa GHBP-like moiety was also labelled by this antibody in both strains (Figure A1.2). Pre-absorption of this antibody with CH17 or replacement with pre-immune serum totally abolished labelling to the 55 kDa and diminished (by >80%) the labelling of the 64 kDa protein (Figure A1.2).

A1.3.B. Binding Studies

After the incubation of K serum with ^{125}I-labelled GH, a single peak of radioactivity was separated on Sephadex G-100 (Figure A1.3). This elution volume of serum was less then that required to elute the 24 kDa monomeric tracer, suggesting that the tracer was binding to a serum protein. The molecular size of GH:GHBP complexes, as determined by comparison with the elution volumes of molecular size markers, was approximately 70-76 kDa, corresponding to a 46-52 kDa GHBP. Tracer incubated with SLD serum eluted at a similar or slightly larger elution volume, thus indicating the presence of serum GHBPs of equivalent or slightly smaller size in SLD serum. This binding protein would also appear to be present in SLD serum in amounts comparable with that in the sera of K birds.

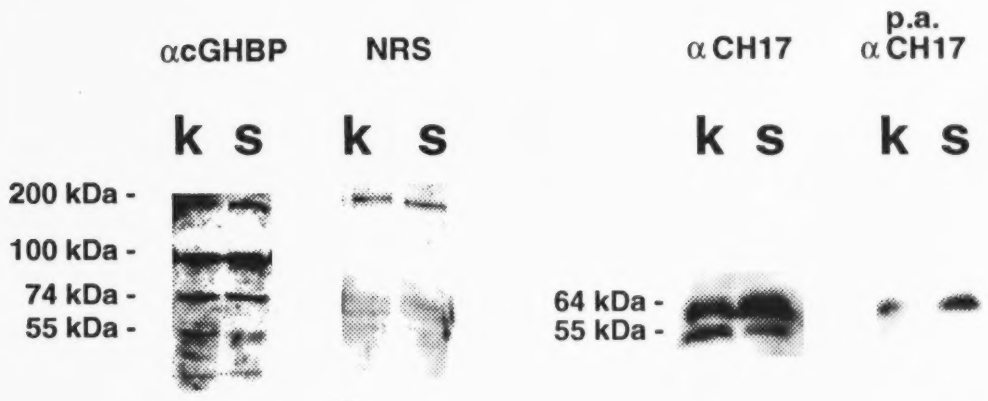

Figure A1.2. Western analysis showing the cross-reactivity of normally-growing (K) and sex-linked dwarf (S) hepatic proteins with polyclonal antibodies raised against recombinant chicken GHBP (αcGHBP) or against CH17 (αCH17). Immunoreactive proteins were visualized using HRP-labelled second antibodies and enhanced chemiluminescence. Identical blots were also incubated with αCH17 that had been pre-absorbed with CH17 (p.a. αCH17) or with normal rabbit serum (NRS).

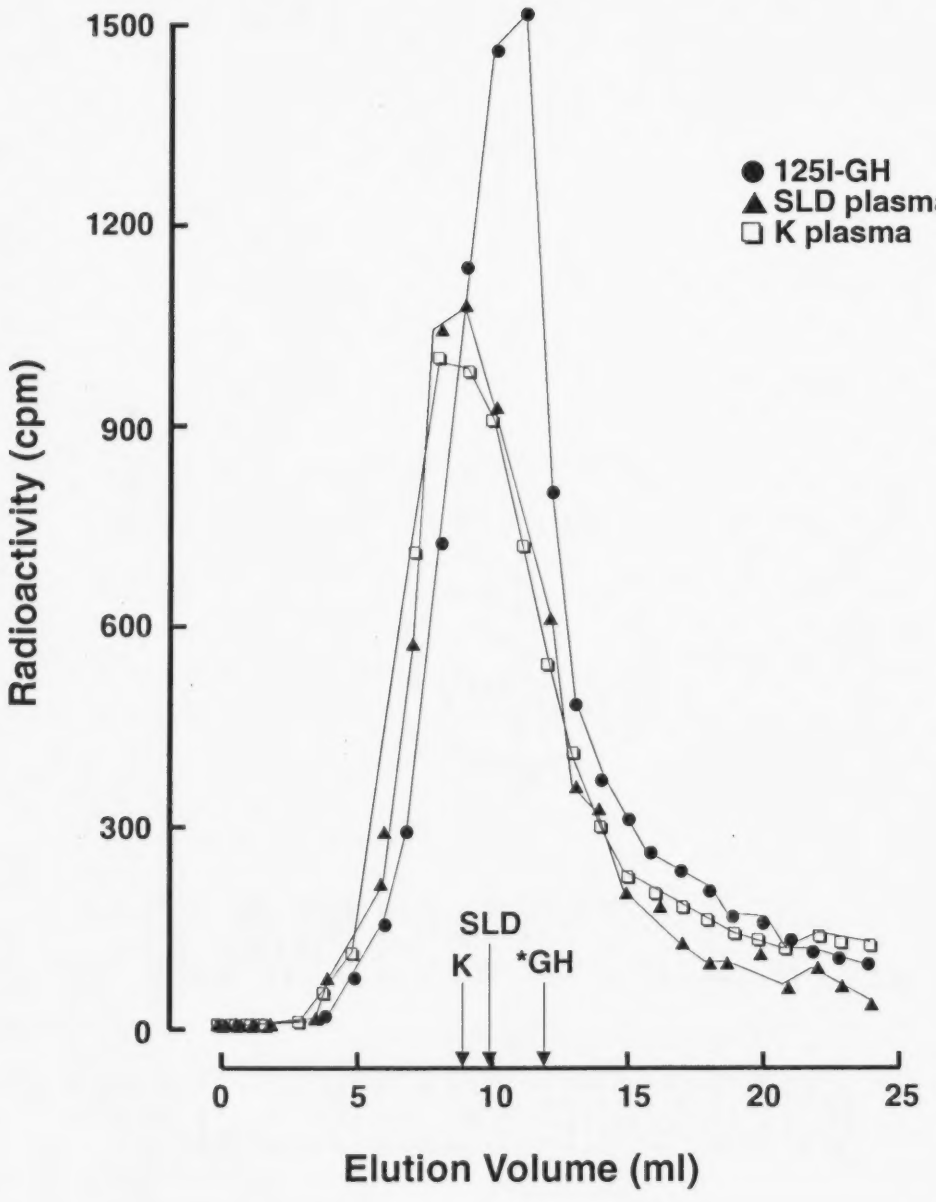

Figure A1.3. Serum GH-binding activity in sex-linked dwarf (SLD) and K (control) chickens. 1 ml aliquots of SLD and K serum were incubated for 2 h at 39C with 1.3 million cpm radiolabelled chicken GH, after which the radioactivity was separated by gel filtration on Sephadex G-100. The elution volumes of incubated sera were less than that required to elute the 24 kDa monomeric GH tracer. The estimated molecular size of both radioactive serum complexes was approximately 80 kDa, based on the elution volumes of molecular size markers.

A1.4. DISCUSSION

These results clearly demonstrate the presence of proteins with GHBP immunoreactivity in the liver and serum of Cornell SLD chickens. These proteins are of identical size to those in normally growing birds and appear to be of comparable abudance. This is an unexpected finding, since Cornell SLDs are GH-resistant.

In view of the putative origins of serum GHBPs (Harvey and Hull, 1994), alternate transcription of the GHR gene may occur in birds, as in rats (Sadeghi *et al.* 1990), mice (Smith *et al.* 1988) and guinea pigs (Hull *et al.*, 1994). The finding of serum GHBP immunoreactivity and radioligand binding sites in Cornell SLD chickens supports this possibility, since these birds are deficent in membrane GH-binding sites. A separate, truncated transcript of the chicken GHR gene that might encode the serum GHBP has, however, yet to be identified. Although transcripts have been detected in chicken tissues that are smaller than that (4.4 kb) coding for the full-length receptor (Hull *et al.*, 1993; Oldham *et al.*, 1993), Oldham *et al.* (1993) showed that a 0.7 kb transcript encoded a truncated receptor rather than a binding protein. A separate GHBP-encoding transcript may thus be of low-abundance or have a rapid rate of turnover.

Although Cornell SLD chickens are deficient in membrane GH receptors, it is, nevertheless, possible that their serum GHBPs may be derived from the full-length receptor as in humans (Leung *et al.*, 1987), rabbits (Leung *et al.*, 1987), and pigs (Bingham *et al.*, 1994) This possibility is supported by the finding of 70 kDa and 100 kDa proteins with GHBP immunoreactivity in Cornell SLD liver homogenates. These proteins are of comparable size to the calculated molecular mass (70 kDa) (Leung *et al.*, 1987) and observed mass (100 kDa) of mammalian GHRs (Yamada *et al.*, 1987), although smaller GH-binding moieties of approximately 70 kDa were detected in some earlier studies (Hughes & Simpson, 1983). It is therefore possible that GHRs are translated in Cornell SLD chickens but these proteins may not be inserted into the plasma membrane or may possess defective binding domains. The etiology of GH resistance in SLD chickens would therefore appear to result from heterogenous lesions, since major gene deletions or defects in transcriptional regulation or post-transcriptional processing are

causal in some populations (Burnside *et al.*, 1991; Burnside *et al.*, 1992; Huang *et al.* 1993). The Cornell SLD strain may also be similar to a Belgian strain (Duriez *et al.*, 1993) in which a mutation in a portion of the gene encoding the extracellular domain of the GHR would impair GH-binding of the translated protein.

The molecular mass of the proteins with GHBP immunoreactivity in Cornell tissues ranged between 45 kDa and 64 kDa and between 70 kDa and > 100 kDa, probably reflecting the presence of binding proteins and receptors, respectively. Earlier studies employing gel chromatography also detected 60-65 kDa GH-binding moieties in hepatic plasma and nuclear membranes and smaller, 55 kDa moities in hepatic cytosol (Hull *et al.*, 1992). While some of these moeities are likely to reflect GHBPs, these indicate that GHRs in avian tissues are smaller than those (100 kDa) detected in mammals. This may partially reflect the absence of exon 3 in the cloned chicken GHR cDNA (Burnside *et al.*, 1991), although hepatic GHR mRNA is of similar size in avian and mammalian livers (Hull *et al.*, 1992). The smaller mass of avian GHRs may thus be due to differential translation or post-translational processing.

The molecular mass of the avian GHBP as determined by gel chromatography (47-52 kDa) differed from that determined by Western Blotting (70 kDa). These results are consistent with earlier observations by Hull *et al.* (1992), who identified 47-52 kDa proteins in serum and hepatic cytosol by gel chromatography, and Vasilatos-Younken *et al.* (1991), who detected 72 kDa GHBPs in fresh serum using cross-linking techniques. This size discrepancy may result from the extended incubation time required for chromatographic analysis, as serum storage increases the appearance of a 52 kDa protein at the expense of the 70 kDa protein in cross-linking studies (Vasilatos-Younken *et al.*, 1991).

In summary, these results demonstrate, for the first time, the presence of GHBPs in SLD chickens and provides information on the possible origins of circulating GHBPs in avian species.

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